The Role of *Drosophila* Bx42/SKIP in Cell Cycle

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Abstract

In order to understand the role of Bx42 in cell cycle regulation of *Drosophila*, I have investigated the effects of the expression of dominant negative Bx42 allele in *Drosophila* eye imaginal discs, as well as the effects of depleting the Bx42 protein in *Drosophila* S2 cells by RNAi. In my study, I found that the expression of Bx42-SNW, a truncated dominant negative version of Bx42, in eye imaginal discs resulted in small and rough eyes. Analyzing the cell cycle in the affected discs showed no significant differences in the number of S-phase cells, but a strong reduction of mitotic cells and the downregulation of Cyc A and Cyc B in the normally mitotic active SMW region of the eye disc. These results are similar to the results of previous Notch signaling studies, which indicated that downregulation of Notch members in *Drosophila* eye resulted in downregulation of Cyc A and Cyc B but not Cyc E and reduction of M-cells. Additionally, Notch overexpression can rescue the Bx42-SNW small eye phenotype. These results suggest that Bx42 is required for cell cycle control in the *Drosophila* eye through its role in Notch signaling. In addition, Bx42-SNW overexpression resulted in elevated apoptosis in the *Drosophila* eye imaginal disc, probably to avoid disruption of development by accumulation of supernumerary non-dividing cells. This study aimed also at finding factors that modify the small eye phenotype resulting from overexpression of Bx42-SNW in the eye. The defined modifiers were E2F/Dp, Rb, Tribbles, Cdk1, Cyclin B3, EGFR, Dpp and Armadillo.

To gain further insight into the role of Bx42 in proliferation, dsRNA-mediated knockdown the expression of Bx42 was employed in S2 cells. Cells treated with dsRNA exhibited a significant decrease in proliferation rates compared to control dsRNA-OFP cells. Cell cycle analysis demonstrated that down-regulation of Bx42 decreased cell populations in the G1& G2 phases simultaneously augmenting S-phase cells by cycle arrest, leading to a state unable to complete cell division. Further experiments to characterize the fate of dsRNA-Bx42 transfected cells indicated that only a minor portion of cells underwent apoptosis or acquired a senescent state. Semi q-RT PCR, revealed that downregulation of Bx42 affects the transcription of E2F, Dap, Cyc A and Cyc B. A reduction of Cyc A and Cyc B was also demonstrated at the protein level. Reduction of E2F, Cyc A and Cyc B following dsRNA-Bx42 transfection might contribute to the observed increase in S phase cells and a decrease G2/M cells. Moreover, downregulation of Dacapo may contribute to a decrease in the G1- and an increase in S-phase population.

Abstrakt

Um die Rolle von Bx42 in der Regulation des Zellzyklus von *Drosophila* zu verstehen, habe ich die Auswirkungen der Expression von dominant negativen Bx42 Allel in *Drosophila* Augenimaginalscheiben untersucht, sowie die Auswirkungen der Bx42 Protein abbau in *Drosophila* S2-Zellen mit Hilfe der RNA-Interferenz-Methode. In meiner Studie fand ich, dass die Expression von Bx42-SNW, einer abgeschnittenen dominant negative Version von Bx42, in den Augenimaginalscheiben zu kleinen und groben Augen führte. Analyse des Zellzyklus in den betroffenen Scheiben zeigte keine signifikanten Unterschiede in der Anzahl der S-Phase-Zellen, aber eine starke Reduktion der mitotischen Zellen und die Herunterregulation von Cyc A und B Cyc in dem normalen mitotischen aktiven SMW Bereich des Augenimaginalscheibes. Diese Ergebnisse sind ähnlich zu den Ergebnissen der vorherigen Notch-Signalweg Studien, die zeigten, dass die Herunterregulation von Notch Mitglieder in der *Drosophila* Augenimaginalscheiben Herunterregulieren der Cyc A und B aber nicht Cyc E und Reduktion von M-phase Zellen führte. Zusätzlich kann Notch-Überexpression die Bx42-SNW kleinen Augenphänotyp retten. Diese Ergebnisse legen nahe, dass Bx42 für Zellzykluskontrolle in den *Drosophila* Augen durch seine Rolle in der Notch-Signalweg erforderlich ist. Außerdem führte Bx42-SNW-Überexpression zu erhöhter Apoptose in *Drosophila* Augenimaginalscheiben, wahrscheinlich zu vermeiden die Störung der Entwicklung durch die Ansammlung von überzähligen nicht teilende Zellen. Ziel dieser Studie war auch Faktoren zu finden, die den kleinen Augenphänotyp von Überexpression der negativen Form Bx42-SNW modifizieren können. Die definierten Modifikatoren waren E2F / Dp, Rb, Tribbles, Cdk1, Cyclin B3, EGFR, Dpp und Armadillo.

Um weitere Einblicke in der Rolle des Bx42 in Proliferation, dsRNA-vermittelte Knockdown der Expression von Bx42 in S2-Zellen verwendet wurde. Zellen, die mit dsRNA behandelt wurden, zeigten eine signifikante Abnahme der Proliferationsraten im Vergleich zu kontrol dsRNA-OFP-Zellen. Zellzyklusanalyse zeigte, dass die Herunterregulation von Bx42 Zellpopulationen in der G1 und G2 Phasen verringerte, gleichzeitig S-Phasen-Zellen durch Zyklusarrest vermehrete, was führte zu einem Zustand, die Zellen unfähig die zellteilung zu vervollständigen. Weitere Versuche um das Schicksal von dsRNA-Bx42 transfizierten Zellen zu charakterisieren zeigten, dass nur ein geringer Teil der Zellen unterzog Apoptose oder einen seneszenten Zustand eintreten. Semi q-RT-PCR ergab, dass die Herunterregulation von Bx42 die Transkription von E2F, Dap, Cyc A und Cyc B beeinflusst. Eine Reduktion von Cyc A und Cyc B auf Proteinebene wurde auch nachgewiesen. Die folgende Reduktion von E2F, Cyc A und B Cyc nachdem dsRNA-Bx42 Transfektion konnte zu dem beobachteten Anstieg der S-Phasen-Zellen und eine Abnahme der G2 / M-Zellen beitragen. Außerdem, Herunterregulation von Dacapo kann zu einer Abnahme in der G1- und einer Erhöhung der S-Phase population beitragen.

1 Introduction

The development of an organism from a single cell to a multicellular three-dimensional structure of characteristic shape and size is the result of coordinated action of genes that direct the developmental fate of individual cells. In order to achieve this, the cells proliferate, differentiate and undergo movements with each of these processes being triggered by different spatial and temporal signaling cues. An important model organism, on which such development processes can be excellently analyzed, is the fruit fly *Drosophila melanogaster*. *Drosophila* has a short generation time, fully sequenced genome and well-studied genetics, which can be deployed to generate mutants and analyze gene function in vivo. Moreover, the giant polytene chromosomes from *Drosophila* larval salivary glands provide an important model system for cytogenetic analyses. In combination with immunological methods, polytene chromosomes have proven to be useful for the study of the distribution of chromosomal proteins with different functions. In a study to identify *Drosophila melanogaster* nuclear proteins, which are involved in the regulation of gene expression, the nuclear protein Bx42 was for the first time identified. Moreover, it was found that Bx42 is localized on transcriptionally active sites (Frasch and Saumweber, 1989; Saumweber et al., 1990). Later, this protein found to be evolutionary conserved and its human homolog the SNW1/SKIP was identified (Baudino et al., 1998; Dahl et al., 1998). Several studies were undertaken in our research group to understand and characterize the roles of Bx42 in the control of gene activity. Using inducible RNA interference Negeri demonstrated that Bx42 is an essential nuclear cofactor involved in Notch signal transduction (Negeri et al., 2002; Negeri, 2003). Furthermore, in her PhD work, El Hachoumi (2007) could show that Bx42 also is involved TGF-ß signal pathway. Investigating the role of different Bx42 protein domains by overexpression, it was shown that the fragment Bx42-SNW has a dominant negative function (Lehmann, 2006; Negeri et al., 2007). By further analysis, it was found that overexpression of Bx42-SNW in the *Drosophila* eye imaginal discs resulted in the reduction of mitotic cells at the second mitotic wave (Negeri unpublished data). This later finding, in addition to the increasing number of studies which relate Bx42 or its homologues to cell proliferation (Boutros et al., 2004; Eggert et al, 2004; Somma et al., 2008; Dahl et al., 1998; Prathapam et al., 2002; Kittler et al, 2004; Bracken et al., 2008) were the main motivations behind this work.

1.1 Bx42: Identification, protein structure and function

The *Drosophila* nuclear protein Bx42 was identified using monoclonal antibodies directed to nuclear proteins (Frasch & Saumweber, 1989). The Bx42 antibody detects the Bx42 protein on polytene chromosomes in about 120 regions of transcriptionally active regions, visible as puffs or prominent interbands (Saumweber et al., 1980; Frasch and Saumweber, 1989). Data from Southern analysis of genomic DNA and in situ hybridization to polytene chromosomes show that Bx42 is present as a single copy gene and it is localized at the X chromosomal locus 8C7-8. The Bx42 gene encodes two transcripts of 1.9 and 2.2 kb, caused by alternative termination of transcription (Wieland et al., 1992). Both Bx42 transcripts encode the same 547 amino acid protein with a molecular weight of 66 kDa. The gene is ubiquitously expressed and transcripts are already detectable in early stages of development. The Bx42 protein can first be detected in the one-hour embryos (Frasch & Saumweber, 1989). This suggests a maternal RNA produced by the oocyte. At the beginning of the nuclear division cycle 13, at the onset of zygotic transcription, Bx42 is detected in all interphase nuclei (Frasch & Saumweber1989).

By analysis of the protein sequence of Bx42 it was found that the protein contains many charged amino acids. The total charge is basic. An accumulation of charged basic amino acid residues occurs in the range between amino acids (aa) 300-400, and between aa 500-547. A predominantly acidic area is located between aa 125-155. A proline-rich region is located between aa 210 and 240. Bx42 does not contain any of the known DNA-binding domains and apart from the SNW domain and a SH2-like domain in the C-terminal half it no other characterized functional domains were found (see below). However, from the comparison of sequence similarities present in Bx42 orthologues three distinct regions can be distinguished (Figure 1-1). First, the N-terminal region contains a strictly conserved motive LPXP and a glycine rich box (reviewed by Folk et al., 2004). Bx42 physically interacts by its N-terminus with the transcription factor Suppressor of hairless Su (H), a component of the Notch signaling pathway. Second, the carboxy-terminal region of Bx42 (aa 493-547) contains a domain that is required for the transactivation activity of the protein (Negeri, 2003). It is highly charged due to the presence of acidic and basic amino acids and harbours a nuclear localisation signal (NLS). Together, the N-terminal parts (aa 1-176) and the C- terminal (aa 493-547) of Bx42 are involved in Bx42 homodimer formation (Negeri, 2003). Third, the central part of the protein potentially forming an alpha-helical coiled coil contains highly conserved motifs consisting of a proline rich box, a SNW signature

(SNWKN, aa 254-257) and a helical repeat. This central region of the protein is called SNW domain and in vertebrates it interacts with the Ski protein (Prathapam et al. 2001a).

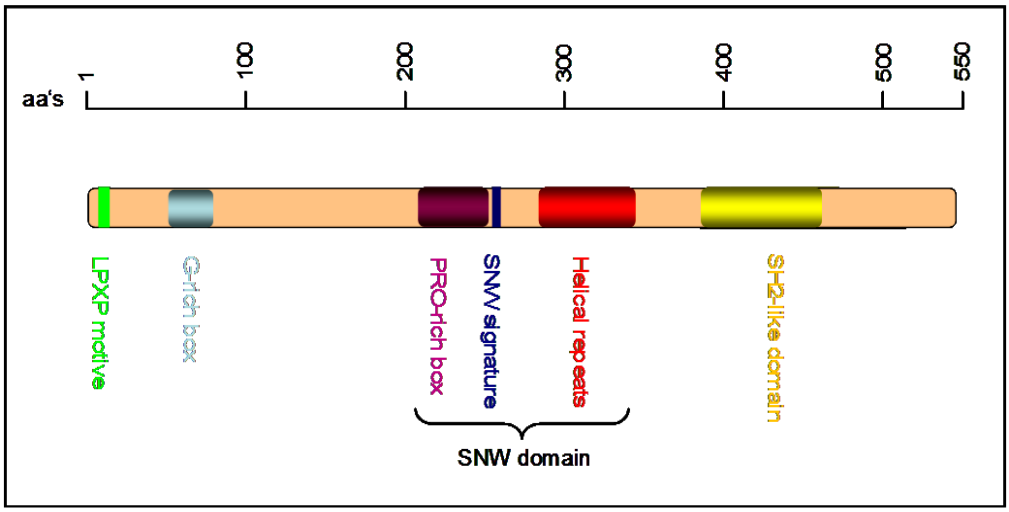


Figure 1-1: Schematic representation of Bx42 domains.
Presented are the highly conserved regions in Bx42. The N-terminus is characterized by the presence of the LPXP motive (aa 7-10) and a glycine-rich box (aa 56-67). The SNW domain consists of a proline-rich box (aa 207-249) which serves as a potential SH3-domain binding site (Folk et al., 1996), the SNW-signature (aa 254-257) and a helical repeat domain (aa 274-333). The C-terminal region is marked by the presence of a SH2-like domain (aa 383-462).

Bx42 is essential for viability, as downregulation of Bx42 in early embryos by RNA interference causes lethality of the embryo (Negeri et al., 2002). In addition, Bx42 was found to be essential for the formation of different tissues during embryo, larval and adult stages of development (Negeri et al., 2002). This is due to the involvement of Bx42 in several major signaling pathways, like nuclear receptor, Notch and TGF-ß/ Dpp signaling.

As mentioned, Bx42 binds to about 120 decondensed sites on polytene chromosomes (Saumweber et al, 1980; Frasch & Saumweber, 1989), amongst them sites responsive to the steroid hormone 20-OH ecdysone. One of these ecdysone-responsive sites is the locus of the *Sgs-4* gene (Saumweber et al., 1990; Wieland et al., 1992). Interestingly, the binding of Bx42 to this site depends on the presence of ecdysone and of a 52 bp sequence within the enhancer region of *Sgs-4*, which is also essential for ecdysone hormone binding. The deletion of the 52 bp sequence resulted in a loss of Bx42 binding at the Sgs-4 locus (Wieland et al., 1992). Later it was found that the vertebrate homolog of Bx42 is an important cofactor for nuclear receptor regulated genes (see below).

Genetic studies in *Drosophila* revealed also the involvement of Bx42 in Notch signaling. It was shown that the effects of Bx42 RNAi phenotypically resemble those obtained for certain classes of *Notch* mutants, pointing to an involvement of Bx42 in the Notch signal transduction pathway. Furthermore, effects on downregulation the Notch

pathway were enhanced by simultaneous loss of Bx42. The wing phenotype following overexpression of Suppressor of Hairless Su (H), a *Notch* loss of function condition, was strongly enhanced by simultaneous Bx42 RNAi induction in the same tissue. After repression of Bx42 or overexpression of Su (H) in the wing imaginal disc, the Notch target genes *cut* and *enhancer of split e (spl) m8* were not expressed anymore (Negeri et al., 2002). Furthermore, it was demonstrated that Bx42 physically interacts with the regulators of *Notch* signaling Su (H) and Hairless. During the Notch activation, Bx42 interacts with Su(H) and Notch-IC and forms a complex that activates Notch target genes (Negeri, 2003).

Evidence in *Drosophila* for an involvement of Bx42 in TGF-ß signaling was first obtained in our group by Bx42-RNAi analysis. The tissue-specific induction of Bx42-RNAi caused a deletion or fusion of leg tarsal segments (Negeri et al., 2002). This phenotype is very similar to the hypomorphic mutant phenotype of the Dpp target gene *distal-less (dll)* (Cohen et al., 1989; Dong et al., 2000; Panganiban, 2000). Later, El Hachoumi could show that Bx42 interacts genetically and in vitro with Mad (dR-Smad), Medea (dCo-Smad) and Smad2 (El Hachoumi, 2007) and controls the expression of several Dpp target genes such as the *optomotor blind* gene in *Drosophila*.

In order to dissect the role of Bx42 /SKIP domains in vivo, Negeri and colleagues (2007) constructed different truncated or mutated versions of Bx42 / SKIP and expressed them in imaginal tissues using the GAL4/UAS system. The overexpression of the full-length Bx42 protein did not affect development. However, ectopic expression of the highly conserved Bx42-SNW domain in different imaginal tissues resulted in phenotypes similar to those obtained following induction of Bx42 RNAi. This indicated that the truncated version Bx42-SNW represents a dominant negative form of Bx42 (Bx42-DN). The ectopic expression of Bx42-SNW in the *Drosophila* eye showed a disruption of ommatidial patterning. This eye phenotype was enhanced by the simultaneous overexpression of the Hairless protein, a repressor of Notch signaling, consistent with the observed interaction between Bx42 and Hairless proteins in vitro. Analysis of the eye imaginal discs of GMR-GAL4/UAS-Bx42-SNW flies indicated a reduction in the number of mitotic cells consistent with a role of Bx42 in cell cycle control (Negeri unpublished data).

1.2 Bx42/SNW/SKIP is an essential protein family, conserved in evolution and involved in several biological processes

Since the identification of Bx42 (Frasch and Saumweber, 1989) homologous genes became identified in other species. The *Fun20*/*Prp45p* gene encoding Fun20/Prp45p of

Saccharomyces cerevisiae (Diehl and Pringle, 1991; Albers et al., 2003) was first identified during analysis of *S. cerevisiae* chromosome I as an essential gene of unknown function. This Bx42-homologous protein lacks a part of the N-terminal Bx42-region that contains the LPXP motive, the glycine rich- and proline rich- box but it still contains the SNW sequence, the helical repeat and the highly charged C-terminal region (Harris et al., 1992). SnwA, the Bx42 homolog of in *Dictyostelium discoideum* was identified in a screen for genes that contain SH2 related domains. Unlike Fun20/Prp45, SnwA is more similar to Bx42 containing the glycine- and proline-rich box at its N-terminal region (Folk et al., 1996). The Bx42 homologue of *S. pombe* SNW1 was published 5 years later (Ambrozková et al. 2001, Skruzný et al, 2001). In between, the human Bx42-homologue *SKIP/NcoA62* (for Ski Interacting Protein, or Nuclear receptor coActivator 62 kDa) was identified (Dahl et al., 1998; Baudino et al., 1998). SKIP/NcoA62 interacts with the cellular and viral forms of the Ski oncoprotein in the two-hybrid system. SKIP shares 60% identity with Bx42 and 40% identity with a *S. pombe* Snw1 homolog. Interestingly, there is a region of 158 amino acids (SKIP aa's 176-333) -the SNW domain- where the identity between the three homologous proteins is 85 - 90% (Dahl et al., 1998). Baudino and his colleagues (1998) identified the same protein as an interaction partner of the vitamin D receptor (VDR) in the yeast two hybrid system. Functional data about the C-terminal part of the SKIP homologous were gained from Snw1, the *Schizosaccharomyces pombe* homolog. This part was found to interact with the small subunit of the splicing factor U2AF (spU2AF23), and involved in homodimer formation (Ambrozkova et al., 2001). In addition, (Baudino et al., 1998) could show that the C-terminus has a transcription activation domain. Snw1 and Prep45 proteins also have the transactivation domain in the C-terminal part (Skruzny et al., 2001; Martinkova et al., 2002). A *Bx42* homolog was also identified in *Caenorhabditis elegans*. CeSKIP is an essential protein, which is expressed ubiquitously at all developmental stages in this species (Kostrouchova et al., 2002). In fox-tapeworm *Echinococcus multicularis*, *Bx42* homolog was called *emSKIP* gene. It encodes a protein of 532 aa's with calculated molecular weight of 60 kDa. By yeast two-hybrid analyses, EmSKIP was also found to form homodimers in vivo. Furthermore, EmSKIP interacts with EmSmadA and EmSmadB, two previously identified TGF-beta/BMP signal transducers of *E. multilocularis*, indicating a role of this protein in TGF-beta signaling processes in the parasite (Gelmedin et al., 2005). In plants, the homolog of human ski-interacting protein in rice was identified OsSKIPa and it was found that it is positively regulates cell viability and stress tolerance (Hou et al., 2009). In addition, *AtSKIP* gene, an *Arabidopsis thaliana* homolog of BX42/SNW/SKIP was identified and

characterized. AtSKIP is expressed at every developmental stage and within the entire plant. Additionally, genome-wide expression analysis revealed that the expression of *AtSKIP* was induced by the hormone abscisic acid, salinity, drought, osmotic stress and cold (Lim et al., 2010). Recently, (Zhang et al., 2013) have identified GmGBP1, a homolog of human ski interacting protein in soybean, and found that it regulates flowering.

Noteworthy, although Bx42 homologues were found in animals, plants and fungi, there is always only one copy of the gene present in the genome. As already noted, sequence comparison of the various Bx42 homologues showed a high similarity score of more than 95% in the SNW domain. This finding suggests that this region might provide an important biological function. As for Bx42 in *Drosophila*, the requirement of the SNW gene family for viability was demonstrated in many different organisms. Firstly, the deletion of *Prp45p/Fun20* causes lethality of yeast cells. Interestingly, the N-terminal half of the protein (aa 1 to 190), was found sufficient to support the essential function in yeast (Martinkova et al., 2002). *CeSKIP* is expressed abundantly in embryos and continues to be expressed in all larval stages up to the adulthood of *C. elegans*. RNAi by *CeSKIP* dsRNA injection into the adult gonads resulted in 100% embryonic arrest between the 50 and 100 cell stage. Treatment of larval animals with *CeSKIP* RNAi resulted in a wide spectrum of postembryonic defects depending on the time and dose of *CeSKIP* RNAi treatment. The most prominent defects included molting defects, larval arrest, defects in movement and body shape irregularities (Kostrouchova et al., 2002). The data of Hou and coworkers (2009) showed that suppression of *OsSKIPa* results in severe growth arrest and eventually death of the plant, suggesting that *OsSKIPa* is essential for maintaining cell viability and normal growth in rice (Hou et al., 2009). Fukuda and colleagues (2002) examined the transcription of SKIP in various tissues of rats and found a high level of expression in several organs including the brain, heart, liver and testis, but low expression in the spleen and skeletal muscle. The broad spectrum of expression of SKIP is in accordance with the previous observations from humans (Baudino et al., 1998), and suggests that this factor is involved in a variety of biological processes. In conclusion, these functional analyses delineate not only the essential function of SNW gene family but also indicate its involvement in several biological processes, which will be discussed below.

1.2.1 Involvement of Bx42/SNW/SKIP in signaling pathways

Signal transduction pathways are responsible for transducing extracellular signals into the cells to modulate their physiological states and gene expression profiles. BX42/SNW/SKIP members were found to be involved in nuclear receptor, Notch and transforming growth factor-β (TGF-β/ Dpp) pathways as will be specified in the following sections.

1.2.1.1 Involvement in nuclear receptor pathways

Baudino and collogues (1998) could demonstrate that the human NCoA-62 formed a direct protein-protein contact with the ligand binding domain of the vitamin D receptor (VDR) and enhanced VDR- mediated gene transcription. Deletion analysis indicated that the highly conserved central region of NCoA-62 is involved in VDR interaction, and the COOH-terminal 50 amino acid region is involved in transactivation. The in vitro interaction between of NCoA-62 and VDR was proven in vivo, where confocal images showed the colocalization of NCoA62 and VDR (Zhang et al., 2003). Chromatin imunoprecipitation (ChIP) identified NCoA-62 on native vitamin D-responsive promoters in osteoblast cells (Zhang et al., 2003; reviewed by MacDonald et al., 2004). The study of Leong and collogues (2004) showed that SKIP bifunctionally modulates (activates or represses) Retinoid-X receptor (RXR) and VDR- dependent gene transcription in a cell line specific manner. SKIP was shown to interact in vitro both with coactivators (steroid receptor coactivator SRC-1 and histone acetyl transferase P300) and with the corepressors N-CoR and SMRT. Kang and coworkers (2010) could show that SIRT1, a NAD+-dependent deacetylase, associates with SKI-interacting protein (SKIP) and modulates its activity as a coactivator of retinoic acid receptor (RAR). Binding assays indicated that SKIP interacts with RAR in a RA dependent manner, through a region that overlaps the binding site for SIRT1. SKIP augmented the transcriptional activation of RAR by cooperating with SRC-1. In contrast, SIRT1 suppressed SKIP/SRC-1-enhanced RAR transactivation activity suggesting that SIRT1 and SKIP play reciprocal roles in the regulation of RAR activity. The androgen receptor (AR) is another member of the nuclear hormone superfamily that is a major player in male sexual development, prostate growth and the in the pathogenesis of prostate cancer. Recently, Abankwa and colleagues found that, SKIP interacts with AR in the nucleus, stabilizes the ligand dependent folding of the AR and augments ligand independent AR transcription (Abankwa et al., 2013).

1.2.1.2 Involvement in the Notch signaling pathway

The Notch pathway provides an evolutionary conserved signaling mechanism that is used by metazoans to control cell fates through local cell interaction. Notch impinges on a wide variety of cellular processes, including the maintenance of stem cells, binary cell fate decisions, differentiation, proliferation and apoptosis (Krejcí et al., 2009; reviews by Artavanis-Tsakonas et al., 1999; Radtke & Raj, 2003; Lai, 2004; Bray, 2006; Borggrefe & Oswald. 2009; Bray& Bernard, 2010). Additionally, Notch signaling interacts with other signaling pathways, such as the EGFR; TGF-β Wingless (Wg) and JNK pathways (Tsuda et al., 2002; Hayward et al., 2005; Müller et al., 2005; Herranz et al., 2008; Protzer et al., 2008; Krejcí et al., 2009; reviewed by Voas & Rebay, 2004; Doroquez & Rebay, 2006; Hayward et al., 2008; Herranz & Milán, 2008).

The Notch -pathway components and Notch activation: The key components of Notch signaling are ligands provided by DSL single pass transmembrane proteins (Delta-type ligands: Delta, Serrate in *Drosophila,* Lag2 in *C. elegans*) that bind the Notch receptor, a single-pass transmembrane protein. Ligand-receptor interaction results in a proteolytic processing of the Notch receptor, splitting off the Notch-intracellular domain (Nicd). Nicd behaves as a transactivator that enters the nucleus and binds to Notch target genes via interaction with transcription factors of the CSL-family (CBF1 in vertebrates, Suppressor of Hairless in *Drosophila*, Lag1 in *C. elegans*). DNA-binding CSL-proteins form a platform on Notch target genes for regulatory protein binding that in the absence of Notch recruits repressor proteins like Hairless and Groucho to keep these genes inactive. In the presence of Nicd the binding of the repressors is competed and replaced by binding of co-activator proteins of the Mastermind family (Mam) resulting in target gene activation (reviewed by Artavanis-Tsakonas et al., 1999; Lai, 2004; Bray, 2006; Edge, 2009; Borggrefe & Oswald, 2009; Kopan & Ilagan, 2009; Artavanis-Tsakonas & Muskavitch, 2010; Guruharsha et al., 2012).

Involvement of SKIP in Notch signaling: The function of human SKIP in the Notch pathway was first demonstrated by Zhou and coworkers (2000 alb). They identified SKIP as a CBF1 binding protein in a yeast two-hybrid screen. Additionally, they could show the interaction between SKIP, and the HDAC and corepressor SMRT. Furthermore, they showed that SKIP also interacts with Notch IC, and is able to bind to N-IC in a CBF1 independent manner. The biological relevance of the interaction between SKIP and N-IC was illustrated by using antisense SKIP mRNA and by mutation of the N-IC ankyrin repeat.

In such experiments, the myoblast differentiation blockage caused by N-IC overexpression was abrogated in the absence of SKIP as well as after the disruption of the SKIP interaction with N-IC. This observation indicates that SKIP is necessary to exercise its function as a coactivator for N-IC. From this study, it was concluded that SKIP could act as an adapter protein for both the N-IC activator complex and the SMRT repressor complex. The SKIP homologs in *C. elegans* and *Drosophila melanogaster* are highly conserved in amino acid sequence. In yeast interaction assays, they found that both the worm and fly SKIP homologs also interact with CBF1/Lag-1 and with NotchIC. This conservation of protein-protein interactions suggests that SKIP is likely to function in Notch signaling analogously across species (Zhou et al., 2000 a).

Notch and the control of the cell cycle: Since in my thesis I will consider the role of Bx42 in cell cycle regulation and since some of the Bx42 functions are mediated by Notch signaling I will briefly introduce to the context dependent roles of Notch in cell cycle control. In the *Drosophila* eye imaginal disc (see below), Notch is required to overcome the G1 cell cycle arrest in the morphogenetic furrow (MF) and trigger the initiation of the second mitotic wave SMW by positively regulating E2F activity and Cyclin A expression, (Baonza & Freeman, 2005; Firth & Baker, 2005; reviewed in Baker,2007 and Carthew, 2007). However, in certain settings Notch signaling is required for cell-cycle arrest. For example, Notch exerts G1 arrest at the zone of non-proliferating cells (ZNC) in *Drosophila* wing imaginal discs by reducing the activity of E2F1 (Herranz et al., 2008). Moderate levels of Notch activation in the wing disc result in increased cell proliferation and reduced cell death (Baonza & Garcia-Bellido, 2000; Giraldez & Cohen, 2003). In another context, Notch signaling causes follicle cells to exit the mitotic cycle and enter the endoreplication cycle through downregulation of *String* during stage 6-7 of *Drosophila* oogenesis (Deng et al.,2001; Lopez-Schier and St.Johnston, 2001). Else, Notch activation causes *String* down-regulation in polar follicle cells, thus inducing the G2 cell cycle arrest (Shyu et al., 2009). A genetic screen for modifiers of the Hairless gain of function phenotype in eye discs (as mentioned, Hairless is a Notch target gene repressor) revealed genes involved in cell apoptosis and cell cycle regulation such as *Dacapo*, *Cyclin B* and *dMyc* (Müller et al., 2005). Moreover, in a genome -wide screen for genes activated by Notch in *Drosophila* cells, Krejcí and coworkers (2009) identified *string* (*stg*, the *Drosophila* homolog of *cdc25*) and *diminutive* (*dm,* the *Drosophila* homolog of *myc*) as Notch target genes. There are in mammals several isoforms of the Notch receptor, which makes analysis more difficult. However, "Notch has two faces; one that promotes and the other that suppresses tumorigenesis. Which of the two faces is shown is

dependent on the cellular context and the crosstalk with other signal-transduction pathways" (reviewed by Radtke and Raj, 2003). In humans, Notch has been found to directly regulate genes involved in proliferation and apoptosis such as *myc*, *Cyclin D, string/CDC25, CDK5,* p21; SEPT4 (Rangarajan et al., 2001; Ronchini and Capobianco, 2001; Jeffries et al., 2002; Klinakis et al., 2006; Palomero et al., 2006; Weng et al., 2006; Joshi et al., 2009; Liu, 2012; reviewed by Koch and Radtke, 2007; Bray and Bernard, 2010). Additionally, the Notch target gene, Hes1 plays essential roles in contact inhibition of cell proliferation in mouse 3T3-L1 cells by repressing *E2F-1* and *Myc*, *Cyclin E1*, and *Cyclin A2* (Noda et al., 2011) and several clinical studies revealed that blocking or activation of Notch signaling in cancer cells affects their proliferation (Li et al., 2010; Hu et al., 2013; reviewed by Olsauskas-Kuprys et al., 2013).

1.2.1.3 Involvement in the TGF-ß/Dpp signal pathway

Transforming growth factor beta (TGF-β) is a member of a large family of multifunctional secreted polypeptides that are potent regulators of cell proliferation, differentiation, as well as extracellular matrix (ECM) production, apoptosis and tumorigenesis (Massagué, 1998; Raftery et al., 1999; Massagué *et al.*, 2005; Tendijke *et al.*, 2000; Derynck *et al.*, 2001). Members of the TGF-β as Bone Morphogenic Protein 2/4 BMP2/4 and its homologue in *Drosophila* Decapentaplegic Dpp, produce different effects, depending on the type and state of the cell. In target cell, this extracellular morphogenic molecule activates the intracellular signal transduction machinery by binding to the two transmembrane serine/threonine kinase proteins known as type I receptor and type II receptor. The binding of Dpp to the type II receptor induces the association of type I receptor to the complex. The activated type I receptor initiates intracellular signaling through the activation of receptor specific R-Smad proteins (Smad-2,-3 for TGF-ß-dependent, Smad -1,-5,-8 for BMP-dependent receptors). R-Smads complex with co-Smads (common Smads) in the cytoplasm and relay the signals into the nucleus where they, together with other proteins, direct transcriptional responses (Massagué, 2000). Interaction between SKIP with Smad2 or Smad3 as well as with the co-Smad protein Smad4 was demonstrated in vivo and in vitro assays. The SNW domain of SKIP seems to be necessary for these interactions. SKIP also augments TGF-ß transactivation in transient transfection assays of mammalian cells (Leong et al., 2001). A direct interaction EmSKIP with the MH2 domain of EmSmadB, the orthologue of Smad1 and Smad5 in humans and of Mad in *Drosophila,* was demonstrated in the fox tapeworm *Echinococcus multilocularis* (Gelmedin et al., 2005). Figueroa and Hayman found that SKIP

has specific inhibitory effects on BMP-2-induced differentiation and implicate SKIP to be a novel regulator of differentiation programming induced by TGF-β signals (Figueroa & Hayman, 2004b). Villar and colleagues (2010) found that in cells stably transfected with SKIP antisense RNA, Smad3 activation decreased, along with an inhibition of epithelial-mesenchymal transition induced by TGF-β1. More recently, it was observed that reduction of SKIP in PC-3 cells enhanced the TGF-β1-induced expression of matrix-metalloprotease 9 (MMP-9), while the ectopic expression of SKIP inhibited MMP-9 promoter transactivation. The effect of SKIP in part may be explained by the reduction of Smad3 activity, which could be necessary to regulate the adequate level of MMP-9. This is supported by reports in which the missense mutations in *Smad3* or depletion of Smad3 in knockout mice showed increased MMP-9 production (Kocić et al., 2012; Villar et al., 2013).

1.2.2 Involvement of Bx42/SNW/SKIP in RNA splicing

Interestingly, a function of Bx42/SNW/SKIP in RNA splicing was found that by enlarge is independent from its role in transcription regulation, although both branches may functionally overlap. Most eukaryotic genes are expressed as precursor mRNAs (pre-mRNAs) that are converted to mRNA by splicing. Pre-mRNA splicing is carried out by the spliceosome, which is an extremely complex macromolecular machine assembled from five uridyl-rich small nuclear RNAs (snRNAs) and over 200 proteins in a highly dynamic fashion (reviewed by Sperling et al., 2008). Interestingly, SNW1/SKIP members were identified as proteins that implicated in splicing process. The human SKIP protein was for the first time implicated in the RNA splicing process, when it was identified in spliceosome by mass spectrometry (Neubauer et al., 1998). Later, it was found that SKIP binds the splicing factor U2AF35 (Ambrozkova et al. 2001). Furthermore, Makarov and colleagues (2002) could show that SKIP is recruited to the spliceosome before the first catalytic step of splicing. It remains bound through the second catalytic step, and still associates with the post-spliceosomal intron complex. The study of (Zhang et al., 2003) mentioned before, proved not only the role of SKIP as transcriptional cofactor for VDR, but also supported a dual role of SKIP as a transcription-splicing coupling factor in VDR-mediated gene expression. They could show that the major class of nuclear proteins interacting with SKIP is components of the U5snRNA. The dual role of SKIP in transcription and splicing was further confirmed in other studies (Nagai et al., 2004; Brès et al., 2005 and reviewed by Brès et al., 2008; Mbonye & Karn., 2014). The human PPIL1 (peptidyl prolyl isomerase-like protein 1) is a specific component of the human 35S-U5 small nuclear ribonucleoprotein particle and the 45S

activated spliceosome. In human colon cancer cell lines it was found that PPIL1 is recruited by SKIP into spliceosome just before the catalytic step 1 and it stably associates with SKIP, which also exists in the 35S and activated spliceosome as a nuclear matrix protein (Obama et al., 2006; Xu et al., 2006).

In *Arabidopsis thaliana*, it was demonstrated that the *Arabidopsis* SKIP physically interacts with the spliceosomal splicing factor Ser/Arg-rich protein45 and associates with the pre-mRNA of clock genes to regulate their alternative splicing and maturation (Wang et al., 2012). In two hybrid system studies, the fission yeast (Prp45p) of SKIP homolog was found to interact with splicing factors U2 auxiliary factor spU2AF and Prp22p (Ambrozkova et al., 2001; Albers et al., 2003). Additionally, C-terminal half of (Prp45p) aids in recruiting Prp22 to the spliceosome and is important for splicing of typical introns (Gahura et al., 2009). Furthermore, Figueroa and Hayman (2004a) could show that SKIP complements the Prp45p deletion and rescues the lethal phenotype.

More recently, Yan and colleagues (2015) could describe the overall structure of the spliceosome and its protein components. Interestingly, Prp45p appears to promote spliceosome assembly by directly interacting with and thus linking together, at least nine distinct proteins and with U2 and U6 snRNAs at the catalytic center. By cryo EM they found that Prp45p contains an extended array of secondary structural elements interspersed by flexible regions that in the spliceosome span a distance of over 150 Aº (Figure 1-2).

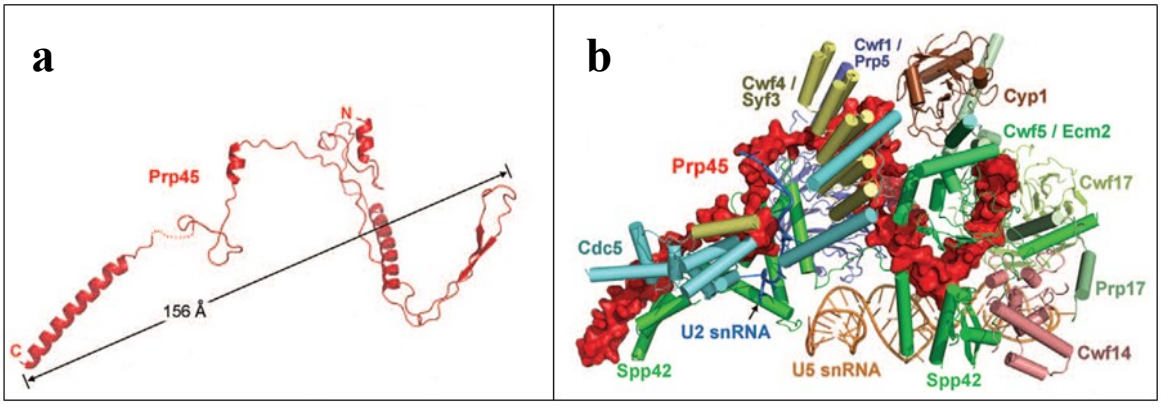


Figure 1-2: Schematic representation of Prp45 structure in the spliceosome
As determined by cryo EM. a) Shows a model of Prp45p in isolation as extracted from cryo EM data of spliceosome. Note the large distance spanned by flexible unstructured elements that allow precise placement of structured parts of the molecule in the complex. . The 216 amino acids in Prp45 span 156 Å. b) same embedded in the spliceosome. The structure of Prp45 is shown in surface view. Prp45 interacts with at least nine protein components and two snRNAs. Data modified from Yan et al., 2015.

They concluded that the morphology and the placement of Prp45p seem to have evolved to facilitate the dynamic process of pre-mRNA splicing. Note the large distance spanned by the unstructured elements that allow flexible placement of structured parts of the

molecule although the N-terminal part is missing, the more canonical Bx42/SNW/SKIP homologues might follow similar rule in folding. This molecular design may explain the functional flexibility of this protein family as an interface between different transcription factors at the promoter or as an adapter molecule between snRNAs and proteins in the spliceosome.

1.2.3 Bx42/SNW/SKIP protein family and cell cycle regulation

1.2.3.1 Short overview on cell cycle regulation.

The cell cycle control system is the regulatory network that controls the steps and timing of cell-cycle events. The central components of the cell cycle control system are two key classes of regulatory molecules, Cyclins and Cyclin-dependent kinases (Cdks) (Figure 1-3) (Nigg, 1995; Morgan, 2007). As the cell progresses through the cycle, decisive changes in the enzymatic activities of the Cdks lead to changes in the phosphorylation state, and thus

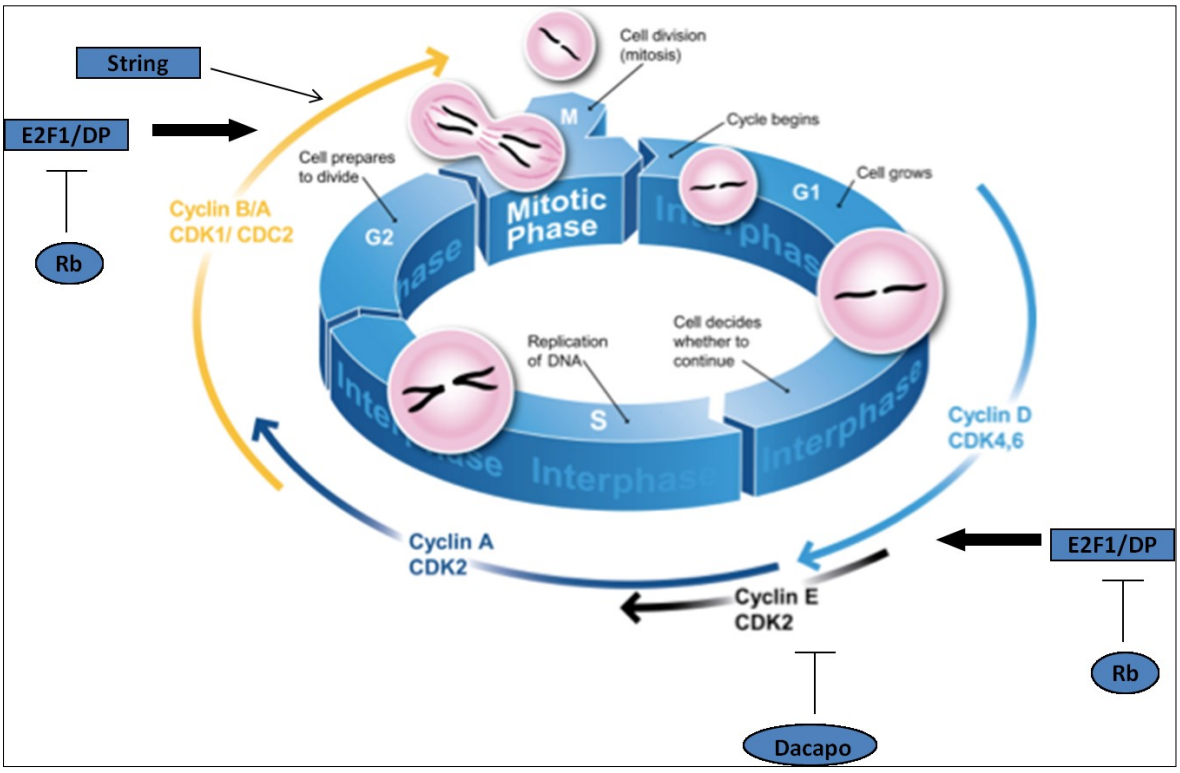


Figure 1-3: A simple representation of cell cycle with the controlling Cyclins-Cdk combinations.
Different Cyclin–CDK complexes regulate progression of cells through the different phases of the cell cycle.

the state of activation, of proteins that control cell-cycle processes. Concentrations of Cdk proteins are constant throughout the cell cycle, oscillations in their activity depend primarily on corresponding oscillations in levels of the regulatory subunits the Cyclins, which bind

tightly to Cdks and stimulate their catalytic activity. Different Cyclin types are produced at different cell cycle stages, resulting in the formation of a consecutive series of active Cyclin-Cdk complexes. These complexes govern distinct cell-cycle events and are referred to as G1-, G1/S-, S-, and M- Cdks (Schafer, 1998; Morgan, 2007).

Besides Cyclin binding multiple regulatory mechanisms govern Cdk activity during the cell cycle. Firstly, full Cdk activity requires phosphorylation by the Cdk-activating kinase (CAKs). In vertebrates and *Drosophila*, the major CAK is a trimeric complex containing a Cdk-related protein kinase known as CdK7, along with its activating partner, Cyclin H, and a third subunit, Mat1 (Morgan,2007). In addition, the Cdk function is regulated by inhibitory phosphorylation by Wee1 (*Drosophila* homologue is Dwee1) and dephosphorylation by Cdc25 (in *Drosophila* called String) **(**Morgan, 1995; Morgan, 1997**)**. Secondly, Cdk inhibitors help suppress Cdk activity in G1, Cdk inhibitor proteins (CKIs) can bind and inactivate Cyclin-Cdk complexes. In *Drosophila* there are two inhibitors one of them is Roughex (Rux) (Thomas et al., 1994; Sprenger et al., 1997; Thomas et al., 1997; Edan, 2000) and the other is Dacapo (Dap) which belongs to Cip/Kip family (de Nooij et al., 1996; Lane et al .,1996), another family of inhibitors in human also is INK4 family. The third mechanism is protein degradation, whereby many cell-cycle regulators are destroyed by ubiquitin-dependent proteolysis (Ou et al., 2003; Ang and Harper, 2005**;** Morgan, 2007). The fourth and last mechanism is the transcriptional control of cell cycle regulators, the E2F family, which cell-cycle-dependently changes in gene expression in metazoans. E2F complexes are heterodimers containing one subunit from the E2F family and one from the DP family. There are at least eight E2F genes and two DP genes in mammals, and two E2F genes and one DP gene in *Drosophila* (Asano et al., 1996; Brook et al., 1996; Du et al., 1996; Frolov et al., 2005; reviewed by Van den Heuvel & Dyson, 2008). The functions of E2F-DP are regulated in part by interaction with members of retinoblastoma pRB family of proteins. These interact with E2F during G1 phase to inhibit the expression of G1/S genes that promote entry into the cell cycle. Lack of the protein pRB promotes unregulated cell proliferation. In addition, pRB proteins are inactivated when they are phosphorylated by G1/Cdks; this phosphorylation triggers dissociation of pRB-E2F complexes, thereby initiating G1/S gene expression and progression through G1 into S phase (reviewed by Van den Heuvel & Dyson, 2008).

1.2.3.2 Evidence for Bx42/SNW/SKIP contribution to cell cycle regulation

It is not surprising, that a multi-functional protein as SKIP might also be implicated in cell cycle regulation. In fact, there are several lines of evidence to suggest such a contribution. **Firstly**, BX42/SNW/SKIP members were found to interact with proteins involved in cell cycle regulation. Human SKIP interacts with its SNW domain with the proto-oncogene Ski (Dahl et al., 1998; Prathapam et al 2001a) that was discovered through its presence in the genome of an avian acutely transforming retrovirus. Endogeneous Ski acts as a transcriptional activator or as a repressor (Stavnezer et al., 1981; Colmenares & Stavnezer, 1989; Nagase et al., 1990). Later Ski was found to interact with the cell cycle protein retinoblastoma (pRb) contributing to transcriptional repression mediated by pRb (Tokitou et al., 1999). Prathapam and coworkers (2002) reported that SKIP interacts with pRb and in cooperation with Ski; it can overcome pRb-induced transcriptional repression. They showed a strong and direct interaction between pRb and SKIP, and they mapped the site of interaction to amino acid residues 171-353 of the evolutionarily conserved SNW domain of SKIP. Additionally, another studies reported a direct interaction among Bx42/SNW/SKIP members and cell cycle factors. Bx42 interacts with microtubule-End Binding 1 (EB1) protein, which is important for proper assembly, dynamics, and positioning of the mitotic spindle. Furthermore, SKIP interacts with FAM53B a protein that needed for cell proliferation (Rogers et al., 2002; Negeri, 2003; Thermes et al., 2006; Kizil et al., 2009; Kizil et al., 2014).

Secondly, several RNAi screen had identified the members of Bx42/SNW/SKIP family as an essential genes for mitosis and proliferation. The *Drosophila Bx42* was identified in an RNAi screen among genes required for growth and viability in *Drosophila* S2 and Kc cells (Boutros et al., 2004). Additionally, using genome-wide RNAi Screens in Kc cells it was found that dsBx42 led to defect cytokinesis, binucleate cells with microtubule extensions (Eggert et al, 2004). Later, another RNAi screen in S2 cells has also identified *Bx42* among *Drosophila* genes required for mitosis, no mitotic division were observed in dsRNAi-Bx42 transfected cells (Somma et al., 2008). Human SKIP was identified also using siRNA screen as an essential gene for cell division in HeLa Human cells. SKIP siRNA resulted of mitotic arrest, cytokinesis defect that led to the formation of cell fragments devoid of chromatin and abnormal spindle formation (Kittler et al, 2004). Other researchers observed an increase in mitotic cells when HeLa cells were transfected with siRNAs for SNW1. However, they did not observe mitotic arrest in either MDA-MB-231cells or MCF7 cells indicating that the

phenotype induced by disruption of RNA splicing may well depend on cell type (Sato et al., 2015).

Thirdly, as splicing factors, Bx42/SNW/SKIP members were also implicated in proliferation process, either directly through mRNA processing of cell cycle factors, or through interaction with other splicing factors required for splicing the cell cycle regulators. SKIP was reported to associate with the SNIP1 complex, which controls Cyclin D1 mRNA stability. SNIP1/SKIP associated RNA- processing complex is coordinately recruited to both the 3′ end of the *Cyclin D1* gene and Cyclin D1 RNA (Bracken et al., 2008). Moreover, Chen and coworkers (2011) could show that the basal and stress-induced p21 expression requires the SKIP/ SNW1 factor. In two human cancer cell lines, U2OS (osteosarcoma) and HCT116 (colon cancer), SKIP selectively regulates p21 pre-mRNA splicing under stress. Recently, scientists provided evidence that SNW1 is essential for sister chromatid cohesion because it is required for splicing of pre-mRNAs that encode sororin and the APC/C subunit APC2 (called ANAPC2). Their data indicated that the main cause of cohesion defects in SKIP-depleted cells is a rapid reduction in sororin levels, which prevents the stabilization of sufficient numbers of cohesin complexes on DNA during S and G2-phase (Sundaramoorthy et al., 2014; Van der Lelij et al., 2014). SKIP also was suggested to modulates genes associated with proliferation or cell cycle progression through its interaction with splicing factor PPIL1 (peptidyl prolyl isomerase-like protein 1; Obama et al., 2006). The role of *Drosophila* Bx42 in splicing is not yet well studied, but it was isolated it as an interaction partner of *Drosophila* MFAP1 protein, which is required for pre-mRNA processing and G2/M progression (Andersen & Tapon, 2008).

The fourth reason is that, Bx42/SNW/SKIP members are cofactors in Notch and TGF-ß signalling. Therefore, they are also implicated in proliferation as members of these signalling pathways (see 1.2.1.2-3). In addition, the members of Bx42/SNW/SKIP family were also implicated in proliferation disturbance during cancer development or treatment, and cell proliferation in tissue regeneration processes and shown to interact with oncogenes and factors implicated in tumour development. SKIP for example interacts with E7, the major transforming protein of human papillomavirus HPV, and with MAGE-A1 (Laduron et al., 2004) and *Drosophila* Bx42 interacts with the human E1A (Adenovirus early region 1A; Prathapam et al 2001b; Negeri, 2003). Other studies showed altered *SKIP* expression in tumor cells or during tumor treatment (Syed et al., 2005; An et al., 2008; Wang et al., 2014), and the data from Olson and his colleagues suggest that a genetic variation in *SKIP* may be associated with risk of breast cancer (Olson et al., 2010).

Liu and coworkers (2013) found that SKIP was overexpressed in the majority clinical HCC (Hepatocellular carcinoma) samples and HCC cell lines. In these cells, SKIP was upregulated during G1 to S phase and SKIP knockdown resulted in a decrease in PCNA protein expression (proliferating cell nuclear antigen, a platform for the assembly of replisomes) that was correlated with a significant decrease in the proliferation rate compared to control cells. Furthermore, cell cycle analysis demonstrated that knockdown of endogenous SKIP decreased cell population in the S phase and augmented cells in G1, indicating a G1 cell cycle arrest. Similar results were found in breast cancer cell lines by Liu and coworkers (2014): the expression of SKIP was up-regulated during the G1 to S phase concomitant with the proliferation marker PCNA whereas p27was decreased; the S phase cell population was diminished and MDA-MB-231 cells were arrested in G1, unable to complete cell division. siRNA-induced SKIP knockdown downregulates the levels of Cyclin A and Cyclin B proteins and changes the expression level of p27.

In another study it was demonstrated that depletion of SKIP induces apoptosis in breast cancer cells (Sato et al., 2015). Chen and coworkers (2013) reported that the expression of SKIP in rat brain cortex subjected to traumatic brain injury was increased along with PCNA and active caspase-3. Similarly, Wu and coworkers (2014) found that SKIP is upregulated in optic nerve at 5 days after optic nerve crush, in parallel with P53 and P21; they also could show the co-localization of SKIP with active-caspase-3 in TUNEL-positive cells under these conditions, indicating that SKIP under certain settings may also contribute to cell death.

In summary, there is substantial evidence for an involvement of Bx42/SNW/SKIP members in cell proliferation. In my thesis, I wanted to investigate this aspect of Bx42 function in *Drosophila.* Since *Drosophila* eye imaginal discs were used in my work as a model, I would like to give a short introduction to the regulation of *Drosophila* eye development in the following section.

1.3 *Drosophila* eye imaginal disc: a model to study proliferation and cell cycle regulation during development.

1.3.1. Overview on eye disc development

Legs, wings, antennae eyes along with dorsal or ventral parts of the corresponding segments in the fly as well as several other adult structures are derived from small sacs of imaginal cells in the larva, called imaginal discs. Each disc originates as a cluster of 10-50 G1-arrested imaginal cells in the embryo. When the larvae begin to feed, these cells grow and divide until the disc contains several thousand cells, which then differentiate into the adult structure during metamorphosis. The imaginal discs are useful for analyzing the role of regulatory proteins in the control of cell proliferation (reviewed by Garcia et al., 2007).

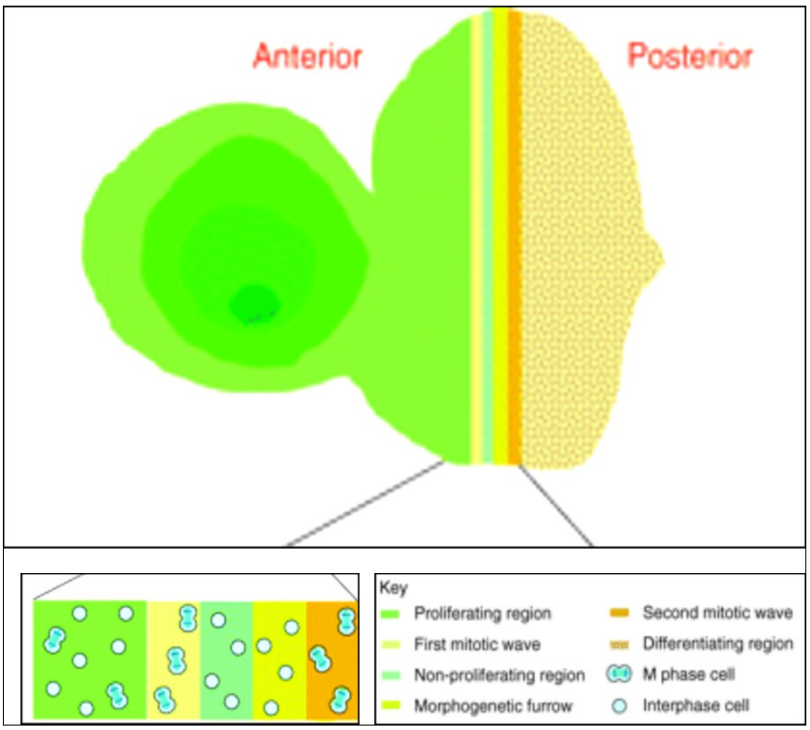


Figure 1-4: Cell cycle progression during development of the eye imaginal disc in *Drosophila* third instar larvae. Well ahead (left in figure) of the morphogenetic furrow (MF), cells progress asynchronously through the cell cycle. Just ahead of the MF, cells become synchronized in mitosis during the first mitotic wave and are then held in G1 in a non-proliferating region. Behind the MF, cells either directly differentiate into photoreceptors or enter a final synchronous S phase, which is followed by a second mitotic wave and subsequent differentiation (Budirahardja & Gönczy, 2009).

A powerful model system in which to address cell cycle control is the developing *Drosophila* eye, in which a regulated terminal mitosis occurs. Retinal development initiates in the monolayer epithelium of the eye imaginal disc during the third larval instar. A wave of development sweeps from posterior to anterior across these cells, preceded by an indentation known as the morphogenetic furrow (MF). Anterior to the furrow, cells proliferate randomly,

and there is no overt differentiation; posterior to it, ommatidial clusters start to form, and the different cells of the adult retina are progressively recruited from the surrounding undetermined cells, reviewed by (Wolff and Ready, 1993, Budirahardja & Gönczy, 2009). Several hours prior the passage of the MF (and therefore anterior to it) all cells in the eye disc arrested in G1-phase, and therefore this region called non-proliferating NPR (Thomas et al., 1994). A few hours later, as they emerge from the posterior of the furrow, cells can be divided into two populations: those in "preclusters" that initiate ommatidial recruitment, and undifferentiated surrounding cells. All cells that have not joined a precluster undergo simultaneously one final round of mitosis, to produce enough cells to complete development. These cells enter S phase and G2, and finally they divide (reviewed in Thomas et al., 1994, and Baker, 2001). This final round of mitosis is called the second mitotic wave (SMW) (Figure 1-4). Because eye development is controlled by an interlocking series of intercellular signaling events, the SMW provides an opportunity to investigate how external signals can control the cell cycle. These generally important pathways include Hedgehog (Hh), Decapentaplegic Dpp, Notch (N), and the epidermal growth factor receptor EGFR.

1.3.2. Signal transduction and proliferation in Eye imaginal disc

1.3.2.1. Hh and Dpp arrest cells in G1

As the MF advances towards, cells in the anterior part of it undergo a first mitotic wave that is synchronized by decapentaplegic (Dpp) and Hedgehog (Hh) signaling (Horsfield et al., 1998; Firth& Baker, 2005; Escudero and Freeman, 2007; reviewed by Baker, 2007). These developmental cues presumably act upon *Stg* expression, as *Stg* RNA is upregulated in these cells in a Dpp- and Hh- dependent manner (Thomas et al., 1994; Escudero and Freeman, 2007). Subsequently, cells arrest in G1 in the non-proliferating region (NPR) through the action of the same signaling molecules. Dpp regulates cell cycle arrest in the anterior part of the NPR through the downregulation of Cyclin E levels and E2F activity (Escudero & Freeman, 2007). Whereas arrest in the posterior part of the NPR is controlled by Hh, through the upregulation of expression of the CKI Dacapo (Dap) and the repression of E2F expression (Thomas et al., 1997; Avedisov et al., 2000; Escudero & Freeman, 2007). Arrest in the posterior part of the NPR is further regulated by the CKI-like protein Roughex (Rux), which targets Cyclin A for degradation and inhibits Cdk2-Cyclin An activity (Thomas et al., 1997; Avedisov et al., 2000 ;). After that, the major player that controls the subsequent G1/S transition is Cdk2-Cyclin E (Sukhanova & Du, 2008).

1.3.2.2. N signaling drives cell cycle reentry in the SMW

Notch signaling plays multiple roles in regulating cell proliferation and differentiation in the developing eye disc (Baker & Yu, 1997; Baonza& freeman, 2001; Li& Baker, 2001; Kenyon et al., 2003). Initially, Notch signaling is required for the upregulation of the proneural gene *Atonal* (*Ato*) (Baonza& Freeman, 2001; Li& Baker, 2001 ;). Notch activity normally only triggers *Atonal* expression in cells that have adopted the pre-proneural state induced by Dpp. Baonza and Freeman reported that Notch drives the transition from pre-proneural to proneural by downregulation two repressors of Atonal: Hairy and Extramacrochaetae (Baonza& Freeman, 2001). Subsequently, Notch signaling is required to limit the number of cells that will differentiate into photoreceptors through a Su (H) dependent process called "lateral inhibition". Consistent with this, while Notch mutant cells block photoreceptor cell differentiation, a majority of the cells within the *Su (H)* mutant clones near the MF differentiate as photoreceptors (Li& Baker, 2001).

Notch signaling was also shown to be required for S-phase entry in the SMW (Baonza& Freeman, 2005; Firth& Baker, 2005). Baonza and Freeman have identified a cell cycle checkpoint in the second mitotic wave and describe how intercellular signaling overcomes this checkpoint. Delta signaling to Notch triggers a progression from G1 arrest in the morphogenetic furrow into the S phase of the terminal mitosis. They have identified two effectors of this Notch requirement, dE2F1 transcriptional activity, and *cyclin A* expression, although their data imply that at least one other target also exists. This is yet unidentified, but they preclude it from being Cyclin E. As high levels of Cyclin E protein were observed in both the *Notch* and the *Su(H)* mutant cells blocked in G1, it was suggested that Cyclin E function was not involved in Notch mediated cell cycle regulation at SMW (Baonza& Freeman, 2005; Firth& Baker, 2005). In another study, Sukhanova and Du (2008) showed that removal of *Su (H)*, a key transcription factor downstream of Notch signaling, blocks G1/S transition in SMW with strong upregulation of Cyclin E/Cdk2 inhibitor Dacapo (Dap). They could show that the upregulation of Dap, which is mediated by bHLH protein Daughterless (Da), is important for cell cycle arrest of *Su(H)* mutant cells in SMW, and that removal of Dap leads to additional cell proliferation and an accumulation of the non-photoreceptor cells in the *Drosophila* developing eye. Interestingly, Delta expression was found to depend on the secreted proteins Hh and Dpp (Baonza and Freeman, 2005).

1.3.2.3. EGFR holds R2-R5 cells in G1 phase and promotes G2/M progression of other cells during the SMW

Cell cycle re-entry in the SMW depends on Notch signaling. This is antagonized by EGFR signaling, which keeps four types of photoreceptor neuron from re-entering the cell cycle R2-R5. Notch activity in other cells drives them into the SMW (Domínguez et al., 1998; Firth& Baker, 2005; Baonza& Freeman, 2005). In this context, the signaling pathways tie differentiation and cell cycle withdrawal together, antagonism between EGFR and N signaling pathways maintain the distinction between cell cycle progression and differentiation. The EGFR activity that promotes fate specification also antagonizes Notch to prevent cell cycle entry, whereas Notch antagonizes cell fate specification and promotes cell cycle entry (Firth& Baker, 2005; Yang & Baker, 2006). Differentiating preclusters in turn activate EGFR in surrounding cells to promote G2/ M progression in the SMW. Baker and Yu (2001) have defined the existence of a checkpoint at the G2-M transition of the SMW. This checkpoint is overcome by EGF receptor (EGFR) activation, which leads directly to the expression of *string*, the *Drosophila* homolog of *cdc25*, (Baker and Yu, 2001 and Baonza et al., 2002; Yang & Baker, 2003). EGFR activation is also essential for survival of second mitotic wave cells (Domínguez et al., 1998; Baker and Yu, 2001).

Lastly, Rbf and Dap withdraw differentiating cells from cell cycle (Firth & Baker, 2005).

It is important here to mention, that Wingless signaling also involved in *Drosophila* eye development, through control of *Drosophila* eye specification "Like the other pathways, Wingless signaling has multiple functions in eye development. In the third instar disc, Wingless expression is restricted to the lateral margin, anterior to the progressing furrow, where it prevents ectopic furrow initiation. Earlier, the localized repression of Wingless by Dpp is responsible for triggering the initiation of the furrow at the posterior margin of the disc " (Baonza and Freeman; 2002). Additionally, the results of Baonza and Freeman (2002) showed that Wingless signaling initiates the border between eye and head, and thereby controls the specification of the retinal territory, by negatively regulating the expression of eye specification genes.

1.4 Non-proliferative state and cell fate decisions

Part of this work included the characterization the fate of cells that cease to proliferate. Non-proliferating cells become quiescent or senescent, or go into apoptosis. Here, I briefly want to summarize the hallmarks of these different fates

Quiescence: Quiescence is a temporary non-proliferating state. Quiescence cells will generally display no DNA synthesis, have a smaller cell size, and a lower metabolism, but under suitable conditions, they can reenter the cell cycle and divide. When scrutinizing the molecular hallmarks of quiescence, one of the first salient findings is the decrease in Cyclin D1 in these cells and overexpression of P130. The CKI that has been rather consistently shown to be active during quiescence is the Cip/Kip family member $p27^{Kip}$, and $p21^{cip}$ (reviewed by Blomen and Boonstra, 2007). Kalamarz and colleagues (2012) found that Dacapo/p21 contributes to progenitor quiescence in lymph gland cells in *Drosophila*.

Senescence: Senescence is a state characterized by the inability to return into the cell cycle. Senescent cells remain metabolically active but display characteristic changes in cell morphology and become big and flattened (Cristofalo et al., 2004),and typically upregulate a senescence-associated β-galactosidase (SA-β-gal) activity (Dimri et al., 1995; Shelton et al., 1999; Reviewed by Campisi, 2001; Campisi & d'Adda di Fagagna, 2007; Campisi et al., 2011; Campisi, 2013). Narita and co-workers could show that senescent IMR90 human fibroblasts accumulate a distinct chromatin structure enriched with heterochromatin proteins, defined as "senescence-associated heterochromatic foci" (SAHF), which excludes active transcription (Narita et al., 2003). Unlike quiescence cells, senescent cells are unable to express genes required for proliferation, even in a promitogenic environment (Dimri et al., 1994, 1996). Mitotically competent cells respond to various stressors by undergoing cellular senescence. These stressors include dysfunctional telomeres, non-telomeric DNA damage, excessive mitogenic signals including those produced by oncogenes (which also cause DNA damage), oxidative stresses or non-genotoxic stress such as perturbations to chromatin organization (reviewed by Campisi & d'Adda di Fagagna, 2007; Campisi et al., 2011; Liu & Xu., 2011; Di Bernardo et al., 2012). The Rb and p53 tumor suppressors are important senescence regulators (Lee et al., 2000; Ferbeyre et al., 2002; Lowe and Sherr, 2003, reviewed by Collins & Sedivy 2003). In many instances, p53 and Rb are activated to promote senescence by products of the *INK4a/ARF* locus (Lundberg et al., 2000; Lowe and Sherr, 2003, reviewed by Collins & Sedivy 2003). Additionally, p21Cip is upregulated with the activation of p53. Cellular senescence in *Drosophila* is not well studied. However, Nakamura and colleagues (2014) could recently show that the senescence machinery also exits in *Drosophila*. Their results indicate that mitochondrial dysfunction causes Ras-activated cells to induce cellular senescence in eye imaginal disc. They showed that, clones of cells expressing oncogenic RasV12 in the eye imaginal disc indeed exhibited several

cellular senescence markers such as elevated (SA-β-gal) activity, upregulation of a Cdk inhibitor Dacapo, elevation of histone H3-K9 trimethylation (which is associated with the senescence-associated heterochromatin foci) and upregulation of heterochromatin protein-1 (HP-1; which binds to methylated K9 of histone H3). Similarly, they found that cells activating Ras signaling in imaginal epithelium showed increased cell size. Their results indicated also that Ras activation and mitochondrial dysfunction cooperate to upregulate p53 activity in these senescent cells.

Apoptosis: unlike the previously discussed cell fates, apoptosis does not allow cell survival. Actually, it is often referred to as "programmed cell death". This is because apoptosis is a highly structured and regulated form of cell death. The principal determinant for apoptosis is the activation of so-called caspases (cys-asp specific proteases). Caspases are present the cytosol in an inactive form (procaspases) and can be activated by external signals ("death receptors" like FasR or TNFαR) or internal signals like the disruption of mitochondria. Upon activation they initiate cleaving and activating effector caspases that, among other properties, will have proteolytic and DNase activity, ultimately leading to cell death. When examining the instigators of apoptosis, one of the best known is p53, which was already discussed for having an important role in senescence. p53 acts by stimulating release of cytochrome c from the mitochondria that together with proteins of the Bcl-2 family stimulates apoptosis due to caspase activation. p53 is often activated in response to stress factors, such as DNA damage and telomere erosion (reviewed by Blomen and Boonstra, 2007). *Drosophila melanogaster* have a single p53 homolog required for DNA damage-induced apoptosis (Brodsky et al., 2004). Intriguingly, expression of the human cell cycle inhibitor p21 or its *Drosophila* homolog Dacapo can suppress both Dp53-induced cell death and differentiation defects in *Drosophila* eyes (Fan et al., 2010).

1.5 Aim of my work

An increasing number of studies implicate proteins of Bx42/SNW/SKIP family in cell cycle regulation. The present study is therefore aimed towards characterizing the role of *Drosophila* Bx42 in cell cycle. To this end, two models were used.

The first model was *Drosophila* eye imaginal disc. Ectopic expression of dominant negative allele of Bx42 was used to induce defects in the proliferation of ommatidial cells. Imaginal discs of of these lines were investigated for cell proliferation and for cell death, and the changes in the expression of candidate cell cycle regulators were investigated. The study was extended to a genetic interaction screen combining candidate loss of function alleles (by mutation or RNAi) or gain of function alleles (by overexpression or gain of function mutations) to identify potential modulators involved in Bx42 mediated cellular proliferation via enhancement or suppression of the Bx42DN eye phenotype.

Secondly, *Drosophila* S2 cells were used as a model to study the effects of Bx42 on cell cycle following knockdown of Bx42 in S2 cells using RNAi knockdown. Effects of Bx42 on cell viability, apoptosis and senesces were examined and changes in timing of cell cycle were studied by FACS analysis to determine the critical phase for Bx42 requirement. The effects of Bx42-RNAi on the expression of candidate cell cycle regulators were investigated by semi q-RT- PCR analyses of the expression of candidate cell cycle regulators at the RNA level and/or protein expression.

By this approach I expected to identify signaling mechanisms whose effects on the cell cycle were mediated by Bx42. I also expected to identify Bx42 target molecules that relayed the Bx42 function to the control of the cell cycle.

2 Materials and Methods

2.1 Materials

All the materials used in this research were purchased from Fermentas, Merck, Boehringer, Ingelheim or Sigma, when there is no reference to another source.

2.1.1 Bacterial- strains

All plasmids were amplified in the *Escherichia coli*-strain XL1-blue with the following genetic markers "recA1 endA, gyrA96 thi-1 hsdR17 supE44 relA1, lac [F′, proAB, lacIqZDM15, Tn10 (Tetr)] c". For growth of bacterial strains the following media were used (for details see Sambrook et al., 1989).

Liquid culture: LB-Medium
Agar plates: LB-Agar
The selection medium contains 200µg/ml ampicillin.

2.1.2 Vectors

Two vectors were used in this work. The first is Spt18 that used for DNA cloning in *E.coli*. The second is Litmus28i used for DNA cloning in *E.coli* for later in vitro transcription of dsRNA. Both vectors use Ampicillin as selection marker when transformed in *E.coli* cells to allow the selection of transformed cells, since they provide their host bacteria with antibiotic resistance.

2.1.3 Primers used in this work

Primers that used in this work are listed in Table **2-1**. Primers were produced by TIB MOLBIOL. Syntheselabor GmbH throughout

Table 2-1: Primers used in this work.

Primer	Sequence(5′-3′)
String forward	ACACCAGCAGTTCGAGTAGCATC
String reverse	CGACAGCTCCTCCTGGTCCAT
Rb forward	CCCGCGTTTTCCATGGCGATT
Rb reverse	GCGATTAGCCAGGACCACACC
Dacapo forward	ACATTGTAACCAATTCGACCAGT
Dacapo reverse	ATAAGGCATCCAAACGTGCAG

Bx42 forward	ACTCGTCCATTTCGCAACTGCT
Bx42 reverse	CCGTCGTCTCCATCACCGTTT
Cyclin A forward	TTGGGCATGGAGATGTGGACG
Cyclin A reverse	TGACACAGATGCAACACAACCG
Cyclin E forward	GATGAGGGCGATGAGACG
Cyclin E reverse	ATTGTTGCTGCTGCTCTTGCCG
Cyclin B forward	TGCCTCACTGTTCCTGTCG
Cyclin B reverse	TAGAAGGTCAGAGTGGGCG
E2F forward	ACCAACAACACTATGGCG
E2F reverse	GTTGTTGATGCTGGTGGTGC

2.1.4 Primary and secondary antibodies used in this work

The antibodies and their related information (Firma, dilution and pin) that were used in this work are listed in Table 2-2

Table 2-2: The primary and secondary antibodies used in this work.

Primary antibodies:			
Antibody	Firma	Dilution	Pin
Primary antibodies:			
anti histone H3 (phospho S10) Mouse monoclonal	Abcam	1:1000	Ab14955
anti Cyclin B / Mouse monoclonal (F2F4-c)	Developmental Studies Hybridoma Bank	1:100	9J2
anti Cyclin A / Mouse monoclonal (A12-c)	Developmental Studies Hybridoma Bank	1:500	9A1
anti Cleaved Caspase-3(Asp175) / Rabbit serum	Cell Signaling	1:2500	#9661
anti Cyclin E (d-300) / Rabbit serum	Santa Cruz	1:300	Sc-33748
anti SNW1/ Rabbit serum	Sigma	1:50	HPA017370
anti Bx42/ Rabbit serum	BioGenes	1:1000	-
anti Bx42/Mouse monoclonal	produced in house	1:1000	-
anti BJ95/ Mouse monoclonal	produced in house		-
anti H2A/Mouse monoclonal	produced in house		-
anti Tubulin/ Mouse monoclonal	produced in house		-
anti Retinoblastoma / Mouse monoclonal	Developmental Studies Hybridoma Bank	1:100	Rb4.1
anti Dacapo/ Mouse monoclonal	Developmental Studies Hybridoma Bank	1:4	NP1

Secondary antibodies:			
Antibody	Firma	Dilution	Pin
anti Alkaline phosphatase-/anti-mouse monoclonal	Jackson Immuno Research	1:2000	115-055-003
anti Alkaline phosphatase-/anti rabbit polyclonal	Jackson Immuno Research	1:2000	111-055-045
Alexa Fluor 488/ anti Mouse	Invitrogen	1:1000	A11017
Alexa Fluor 488/ anti Rabbit	Invitrogen	1:1000	A11070
Alexa Fluor 500/ anti Mouse	Invitrogen	1:1000	A21422
Alexa Fluor 555/ anti Rabbit	Invitrogen	1:1000	A21428

2.1.5 Fly strains

The wild type reference strain used in this work was *Oregon R*
The transgenic lines used in this work were purchased from the Bloomington stock center and are listed in Table 2-3:

Table 2-3: the transgenic lines.

Cell cycle control system			
Stock number	Gene	Mutant	Chromosome
6642	*Cdk1, (cdc2)*	UAS Cdk1	2
6641	*Cdk1, (cdc2)*	Cdk1 loss of function	2
6636	*Cdk2, (cdk2)*	Cdk2 loss of function	3
6634	*Cdk2, (cdk2)*	UAS Cdk2	3
6631	*Cdk4*	UAS Cdk4	2
6633	*Cyclin A* (*Cyc A*)	UAS Cyc A	2
27718	*Cyclin D (CycD)*	Expresses dsRNA for RNAi of Cyc D (FBgn0010315) under UAS control	3
13203	*tribbles (trbl)*	trbl loss of function	3
4781	*Cyclin E (Cyc E)*	UAS Cyc E	3
30725	*Cyclin E (Cyc E)*	UAS Cyc E	2
10384	*Cyclin E (Cyc E)*	Cyc E hypomorphic	2
6626	*Cyclin B (Cyc B)*	UAS Cyc B	2
6630	*Cyclin B (Cyc B)*	Cyc B loss of function	2
6628	*Cyclin B3 (Cyc B3)*	UAS Cyc B3	2
6635	*Cyclin B3 (Cyc B3)*	Cyc B3 loss of function	3
4557	*Cdk7*	Cdk7 loss of function	1

6639	*Dacapo (dap)*	dap loss of function	2
11377	*Dacapo (dap)*	dap loss of function	2
3499	*wee*	wee hypomorphic	2
4777	*string (stg)*	UAS stg	2
9166	*roughex (rux)*	UAS rux	2
9165	*roughex (rux)*	rux loss of function	1
2492	*fizzy (fzy)*	fzy loss of function	2
11717	*E2F transcription factor(E2f)*	E2F loss of function	3
5553	*DP transcription factor (Dp)*	Dp hypomorphic allele	2
7435	*Retinoblastoma-family protein (Rbf, Rb)*	Rb loss of function	1
16803	*Retinoblastoma-family protein (Rbf, Rb)*	Rb loss of function	1
8342	*Retinoblastoma-family protein (Rbf, Rb)*	Rb loss of function	2
4774	*E2F, Dp*	UAS E2F N, UAS Dp D	2
31501	*Archipelago (ago)*	Expresses dsRNA for RNAi of ago (FBgn0041171) under UAS control.	3
16989	*Archipelago*	loss of function	3
Play role in eye development			
1486	*decapentaplegic (dpp)*	UAS dpp	3
2062	*decapentaplegic (dpp)*	dpp hypomorphic allele	2
2070	*decapentaplegic (dpp)*	dpp ,hypomorphic allele	2
5364	*Epidermal growth factor receptor (EGFR)*	UAS EGFR DN	2,3
2079	*Epidermal growth factor receptor (Egfr)*	Egfr hypomorphic allele	2
3378	*armadillo (arm)*	arm loss of function	x
4782	*armadillo (arm)*	UAS arm	x
5612	*Delta (Dl)*	UAS Dl	1
Genes that are components of the contractile ring			
11215	*zipper*	zip loss of function	2
25712	*spaghetti squash (sqh)*	sqh loss of function	1
27616	*diaphanous (dia)*	UAS dia	3

4892	*chickadee (chic)*	chic loss of function	x
9234	*twinstar (tsr)*	UAS tsr N	1
Organize and regulate the contractile ring			
11194	*peanut (pnut)*	pnut loss of function	2
4366	*sticky (Sti)*	Sti hypomorphic	3
6671	*Rho-kinase (rok)*	UAS rok	3
11089	*Stretchin-Mlck (Sttrn-Mlck)*	Sttrn-Mlck loss of function	2
11607	*Myosin binding subunit (Mbc)*	Mbc hypomorphic	3
Genes that are important for completion of mitosis			
16103	*Pimples (pim)*	pim loss of function	2
18610	*separase (Sse)*	Sse loss of function	3

The RNAi lines used in this work were purchase from VDRC (Vienna *Drosophila* RNAi Center) Table 2-4:

Table 2-4: the RNAi lines purchased from VDRC used in this work.

CG Nr	Stock	Hairpin Length	FlyBase Genotype
CG5940	Cyclin A RNAi	364	P{KK101548}VIE-260B
CG6376	E2f RNAi	412	P{KK100304}VIE-260B
CG7413	Rb RNAi	343	w^{1118}; P{GD4484}v10696
CG 1395	string RNAi	321	w^{1118}; P{GD8177}v17760/TM3
CG3510	Cyclin B RNAi	361	w^{1118}; P{GD10993}v43772
CG4488	wee RNAi	316	P{KK109180}VIE-260B
CG5408	trbl RNAi	352	P{KK108667}VIE-260B
CG4654	Dp RNAi	302	w^{1118}; P{GD4444}v12722

2.2 General molecular biological methods

2.2.1 The synthesis of recombinant plasmid:

2.2.2 Polymerase chain reaction (PCR)

Polymerase chain reaction provides a basis to amplify DNA fragments.

PCR is performed using the following standard reaction mixture:

Template DNA	1 µM
10X amplification puffer	5 µM
Mixture of dNTPs	2 µM
Primer1	2µM (≈100pmol)
Primer2	2 µM (≈100pmol)
Taq DNA polymerase	1 µM

H_2O to a final volume of 50 µl

The standard conditions for PCR amplification were:

94 ºC 2min

Denaturation 94 ºC 20 sec

Annealing 50– 65 °C (according to the primer annealing temperature) 30 sec } ×30 cycles

Elongation 72 ºC 01:30 min

72 ºC 10 min

The reaction was regularly performed in a 30 cycles.

2.2.3 DNA ligation

Joining linear DNA fragments together involves creating a phosphodiester bond between the 3' hydroxyl of one fragment and the 5' phosphate of another fragment by the ATP- dependent T4 DNA ligase. This enzyme will ligate DNA fragments having overhanging ends and fragments with blunt ends. The molar ratio used was 1:2 of cut vector to the DNA-fragment to be inserted. The ligation reaction occurred over night at 16°C in water bath.

The components of the ligation reaction were:

10X T4 DNA Ligase Buffer 2µl
Vector DNA: Insert DNA in 1:2 ratio
T4 DNA Ligase 1 µl

Nuclease-free water up to 20 µl

2.2.4 Bacterial Transformation

2.2.4.1 Plasmid Transformation and Antibiotic Selection

For Plasmid transformation, 2µl of plasmid DNA was added to 50µl of XL1 bacteria cells and the tubes were stored on ice for 30 min. The cells were then heat shocked at 42 °C for 2 min. Then they were rapidly transferred to an ice bath allowing the cells to cool for 2 min. Then 200µl of preheated LB medium were added to the cells, which were then incubated 30 min at 37°C for 45 min to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. The appropriate volume (100-150µl) of transformed competent bacterial cells was transferred on an agar LB medium plate containing the appropriate antibiotic. The plates were incubated overnight at 37 °C, and at the next day, the isolation of plasmid DNA was performed.

2.2.5 Preparation of Plasmid DNA by Alkaline Lysis with SDS - minipreparation

Plasmid DNA is isolated from small- scale (1-2ml) bacterial cultures by treatment with alkali and SDS. One single colony of transformed bacteria was incubated in 2 ml LB-Medium containing the appropriate antibiotic overnight at 37 °C with vigorous shaking. The next day 1,5ml of the culture was transferred into a microfuge tube and centrifuged at 13000 rpm for 1 min. Then, the supernatant was removed from the microfuge tube. The bacterial pellet was resuspended by vigorous vortexing in 100µl of ice-cold alkaline lysis solution I. Then 200µl of freshly prepared alkaline lysis solution II was added to the bacterial suspension and the tube contents were mixed by inverting the tube rapidly 5 times.

The tube was stored on ice, 150µl of ice-cold alkaline lysis solution III was added, and the tube was inverted several times and then stored on ice 5 min. The bacterial lysate was centrifuged at 13000 rpm for 10 min and the supernatant transferred to a fresh tube. Then 800µl of 96% ethanol was added, mixed by vortexing and the mixture was allowed to stand 10min on ice. The precipitated plasmid DNA was collected by centrifugation (13000 rpm for 10) min and the supernatant was removed. Then it was washed with 500µl 70% ethanol and the tube centrifuged at 13000 rpm for 5min. After that, the supernatant was removed and the tube was allowed to stand in an inverted position on a paper towel to drain all of the fluid. The nucleic acids were dissolved in 50µl of TE puffer (pH 8,0) and stored at -20°C.

Solution I: 50 mM Glucose, 25mM Tris HCl (pH 8), 10 mM EDTA (pH8)

Solution II: 0,2 N NaOH, 1%SDS

Solution III: 3 M Potassium acetate, 5M Acetic acid

2.2.6 Preparation of Plasmid DNA using QIAfilter plasmid Midi kit (QIAGEN)

A single colony from a freshly streaked plate was picked and incubated in 50 ml LB medium containing the appropriate selective antibiotic at 37°C for12-16 hour with a vigorous shaking. Cells (30 ml) were collected by centrifugation at 6000xg for 15 min at 4°C. Pellet was resuspended in 4ml of buffer P1 and cells were lysed by adding 4ml buffer P2, mixed thoroughly by vigorously inverting the sealed tube 4-6 times, and incubated at room temperature (15-25°C) for 5min. 4ml of pre-chilled buffer P3 were added to lysate and mixed thoroughly by vigorously inverting 4-6 times. Lysate was transferred into a reservoir of the QIAfilter Cartridge and incubated at RT up to 10 min. Buffer QBT was applied to a QIAGEN-tip 100 and the column was allowed to empty by gravity flow. The cap was removed from the QIAfilter cartridge outlet nozzle. The plunger was gently inserted into the QIAfilter Midi cartridge and the cell lysate was filtered into the previously equilibrated QIAGEN-tip. The filtered lysate was applied onto the QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed with wash Buffer QC and DNA was eluted with buffer QF and the flowthrough was collected. DNA was precipitated by adding 3,5ml room temperature isopropanol to the eluted DNA. After mixing and immediate centrifugation for 30 min at 4°C and 15000x*g* DNA pellet was washed with room-temperature 70% ethanol, and centrifuged for 10min at 15000 x *g.* The pellet was (5-10 min) air-dried and the DNA was redissolved in a suitable volume of TE buffer.

2.2.7 RNAi methods

2.2.7.1 Preparation of templates by PCR to be used for in vitro transcription

Suitable DNA templates can be generated by cloning fragments into LITMUS 28i/38i vectors (Figure 2-1), where they can be amplified using a single T7 promoter-specific primer, generating double-stranded DNA templates whose ends are defined by the T7 promoters themselves. The template for transcription can be prepared by PCR with the included T7 promoter primer, which will hybridize to the T7 promoters on either side of the cloned insert and amplify any DNA between them.

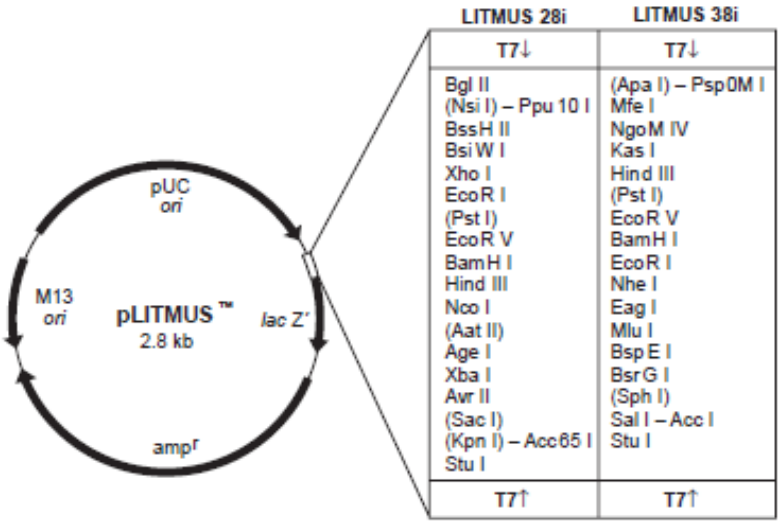


Figure 2-1: LITMUS vectors for dsRNA production.

PCR is performed using the following standard reaction mixture:

Template DNA	100 ng
10 × Green Dream Tag puffer	5µl
Mixture of dNTPs	1µl
T7 Primer	1,5µl
Taq DNA polymerase	0,5µl
H_2O to a final volume of	50 µl

To get a better DNA quality, second PCR was done using the following standard reaction mixture:

Template DNA	100 ng
10 × Dream Tag puffer	5µl
Mixture of dNTPs	1µl
T7 Primer	1 µl
Taq DNA polymerase	0,5µl
H_2O to a final volume of	50 µl

The following PCR program was used:

94ºC 2min

94ºC 20 sec
45ºC 30 sec
72ºC 01:30 min
} ×30 cycles

72ºC 10 min

The PCR product then was purified by ethanol precipitation. The quality and the quantity of the PCR product can be tested by gel electrophoresis (1,2% agarose-TAE buffer).

2.2.7.2 In vitro transcription

The following reaction mixture was used:

5X Transcription buffer	10µl
ATP/GTP/CTP/UTP Mix, 10mM each	10µl (2mM final concentration)
DNA template	1µg
RiboLock RNase Inhibitor	1,25µl
T7 RNA Polymerase	1,5µl

RNase-free water to 50 µl total volume

This mixture was incubated at 37 ºC overnight. At the next day 2µl DNase was added to remove the DNA template, mixed and incubated at 37 ºC for 30 min. Then the dsRNA was precipitated.

2.2.7.3 RNA Precipitation with Ammonium Acetate

Ammonium acetate solution was added to the transcription reaction to a final concentration of 2,5M. The mixture was chilled on ice for 15min, and was centrifuged in a microfuge at 13000 rpm for 30min. The supernatant was removed and the pellet was washed with 70% ethanol to remove salts. The RNA pellet was resuspended in a desired volume of RNase-free water and was quantitated.

2.2.7.4 RNAi treatment of cells

1ml serum-free and antibiotic-free *Drosophila* Schneider's medium was added in each well of 6 well plates, then 1×10^6 S2 cells and the desired amount of dsRNA were added to it. The plate was swirled slowly to produce uniform distribution and was incubated at room temperature for one hour. After that 2ml serum -containing medium was added to each well, the plate was swirled, and placed in the incubator at 25 ºC until analysis.

2.2.8 Semi q RT-PCR

2.2.8.1 RNA Isolation

Cells were spun down for 5min at 300× rpm. The medium was removed and the cells were lysed with TRIZOL (PeQLab Technology) reagent by repetitive pipetting. 1 ml of reagent per 5-10×10^6 cells was used. The homogenized sample was incubated for 5min at room temperature to permit the complete dissociation of nucleoprotein complexes. 0,2ml of chloroform per 1ml TRIZOL reagent was added. The sample tubes were caped securely and the samples were vortexed vigorously for 15 sec and incubated at room temperature for 2-3

min. The samples were centrifuged at 13000 rpm for 15min at 4 ºC. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The upper aqueous phase was carefully transferred into fresh tube. The RNA was precipitated from the aqueous phase by mixing with 0,5ml isopropyl alcohol. The samples were incubated at room temperature for 10 min and centrifuged at 13000 rpm for 25 min at 4ºC. The RNA pellet was washed after removing the supernatant completely with 1 ml 75% ethanol. The samples were mixed by vortexing and were centrifuged at 13000 rpm for 25 min at 4ºC. The RNA pellet was air dried for 5-10 min. The RNA was dissolved in DEPC-treated water by passing solution a few times through a pipette tip (Chomczynski and Mackey, 1995).

2.2.8.2 DNase Treatment

The genomic DNA was removed from the RNA sample using the following mixture:

RNA in DEPC-treated Water	10µl
10×reaction buffer with $Mgcl_2$	1µl
DNase I, RNase-free	1µl

The mixture was incubated at 37 ºC for 30 min. A

Thereafter 1 µl 50mM EDTA was added, and the RNA sample was incubated at 65 ºC for 10 min.

2.2.8.3 First Strand cDNA Synthesis

The prepared RNA was used as a template for reverse transcription using the following mixture in the indicated order:

Template RNA	12µl
Oligo $(DT)_{18}$ primer	1µl
Random Hexamer primer	1µl
dNTPs Mix, 10 mM each	1µl (0,5mM final concentration)
5× RT Buffer	4µl
RiboLock RNase Inhibitor	0,5µl
RevertAid Premium Reverse Transcriptase	1µl

The mixture was mixed gently and was centrifuged briefly. Then it was incubated at 50 ºC for 30 min, then at 85 ºC for 5 min. The cDNA was stored at -20°C for further analyses.

1µl of the cDNA was used to perform qRT-PC. The PCR program for semi qRT-PCR reaction was:

94ºC 2min

94ºC 15 sec
57ºC 15 sec
72ºC 30 sec
} ×30 cycles

72ºC 10 min

and using the PCR Machine: Biorad PCR-Cycler iCycler (Figure 2-2).

Figure 2-2: Biorad PCR-Cycler iCycler PCR Machine

2.2.9 FACS Analysis

To prepare cells for FACS analysis, 1-3 million cells were washed twice with cold sterile 1x PBS. Then, cells were resuspended in 0,5ml cold sterile 1X PBS and 5 ml of 95% ethanol were slowly added and the cells were stored until analysis. The day before analysis cells were washed with 3 ml cold sterile 1x PBS. Thereafter, the cells were resuspended in 1 ml cold sterile 1x PBS, and RNase A and propidium iodide were added to a 1x final concentration and stored at 4ºC overnight. At the day of analysis, the samples were warmed to room temperature, and transferred to a 6-mL tube suitable for flow cytometric analysis (Dahmann, 2008). Stained cells were analyzed with a BD FACS Aria II cell sorter (BD FACS Aria II) (Figure 2-3), and the data were analyzed using De Novo- FCS 4 Express flow plus image software.

20X Propidium iodide: 0,5 mg/mL propidium iodide in 38mM sodium citrate.

40X RNase A: 100 µg/ml DNase-free RNase A, 10mM PIPES, pH 6.8, 2mM $MgCl_2$, 0.1 M NaCl, 0.25mM EDTA, and 0.2% Triton X-100.

95% Ethanol: filter-sterilized 95% ethanol in dH_2O.

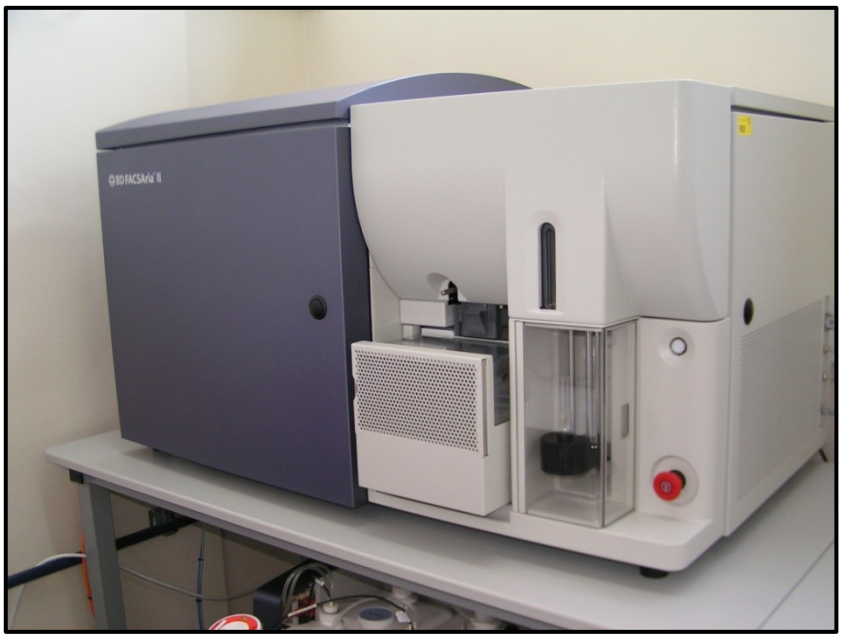


Figure 2-3: BD FACS Aria II

2.2.10 SDS- Polyacrylamide Gel electrophoresis (SDS-PAGE)

The proteins are separated in accordance to their molecular weight in a SDS-PAGE, this occurs with appropriate resolving gel (Sambrook et al., 1989). The appropriate resolving gel was poured into the gap between the glass plates. The gel was overlaid using a pipette with 0.1 % SDS solution. After polymerization was complete (circa 1 h), the 0.1% SDS was poured off and the stacking gel mixture was added onto the surface of the resolving gel, the appropriate comb was placed into the stacking gel. The stacking gel was left to polymerize (circa 1h). The gel was placed into the electrophoresis chamber and the chambers were filled with electrophoresis buffer and the comb was removed from the stacking gel. The proteins were dissolved in SDS loading buffer, the samples were heated at 95°C for 10 minutes and were loaded onto the bottom of the wells. After gel running is finished, the proteins could be visualized by Coomassie staining or western blot.

Electrophoresis buffer: 25 mM Tris
192 mM glycine (electrophoresis grade) pH 8.3
0.1 % SDS
0.2

SDS loading buffer:	1.25 ml 1 M Tris-HCl pH 6.8
	2 ml 20% SDS
	2 ml 87 % glycerol
	1 ml mercaptoethanol
	60µl bromophenol blue

2.2.10.1 Coomassie staining

The gel was washed with water 3×10 min, then it was fixed and stained with 20 ml Coomassie staining solution for 1 hour to overnight. Afterwards the gel was destained by washing 3×5 min with pure water to visualize the band.

Coomassie staining solution: Page Blue Protein Staining Solution from Fermentas (#R0571), store at 4-26°C.

2.2.10.2 Western blot

The proteins from the SDS-PAGE were electrophoretic transferred into a nitrocellulose transfer membrane for 2h at 0.4 mA in blot buffer. Subsequently the membrane was transferred to block buffer and was blocked for 1h under shacking, then was incubated overnight with the first antibody at 4 °C. After wash step (3×10 min with PBS) the membrane was incubated for 4 hours with an alkaline Phosphatase- coupled antibody (1:2000 in block buffer). The membrane was then incubated with NBT buffer for 20 min. The detection of the proteins was occurred by adding the substrate NBT/BCIP (in NBT buffer) to the membrane.

Blot buffer:	25 mM Tris-Base
	192mM glycine
Block buffer:	1 % BSA
	1 x PBS
NBT buffer:	10 mM Tris-HCl pH 9.5
	100 mM NaCl
	10 mM $MgCl_2$

2.3 Working with flies

2.3.1 Acridine orange staining

Imaginal discs from third in star larvae were prepared and collected in ice cold PBS then stained for 5 min in 1µg/µl acridin orange in PBS. Discs were then rinsed briefly three times with PBS, mounted in PBS and immediately viewed by fluorescent microscopy.

2.3.2 Immunostaining of Imaginal discs

Imaginal were dissected from third instars larvae and collected in ice cold PBS. The dissected material was then placed in fixative solution for 30 min at RT. After fixation, the discs were washed with PBT two times short and three times for 10 min each. Then in block solution for 30 min at RT to prepare the sample for immunostaining. The discs were then incubated overnight at 4 °C with the primary antibody, which was diluted in block solution. The imaginal discs were washed the next day with PBT three times each 20 min. The discs were then incubated 2 hours at RT, protected from light, with the secondary antibody, which was diluted in block solution. The imaginal discs were washed three times each 20 min with PBT, once with PBS, then were stained with Hoechst 1:5000 in PBS for 30 min, and were mounted in glycerol Propylgallat before imaging by fluorescent microscopy.

PBS: 130mM NaCl, 7mM Na_2HPO_4 , 3 mM NaH_2PO_4 ,pH 7.4
PBT: 130mM NaCl, 7mM Na_2HPO_4 , 3 mM NaH_2PO_4 ,pH 7.4
0.3 % v/v Triton X-100
Fixative solution: 4 % formaldehyde in 1 x PBS
Block solution: 5 % FCS in 1 x PBT

2.3.3 EdU incorporation

The EdU kit from Invitrogen was used to visualize S-phase cells. EdU (5-ethynyl-2´-deoxyuridine) provided in the kit is a nucleoside analog of thymidine and is incorporated into DNA during DNA replication.

2.3.3.1 Labeling cells with EdU

Imaginal discs were dissected from third instars larvae and collected in Schneider's medium. Then the discs were incubated with 1ml of 40µM of prewarmed EdU in Schneider's medium for 90 min at RT.

2.3.3.2 Fixation and permeabilzation

After incubation, the solution was removed and 1ml 3.7% formaldehyde in PBS was added for 30 min at RT. Then, the fixative was removed and the discs were washed twice with 3%BSA in PBS 5min each. The discs were permeabilized by adding 1ml of 0.5 %Triton x-100 in PBS for 20 min at RT.

2.3.3.3 EdU Detection

The permeabilzation buffer was removed and the imaginal discs were washed twice with 3% BSA in PBS 5min each. Then 0.5 ml Click-iT reaction was added and the discs were incubated for 30min at RT, protected from light and washed with 3%BSA in PBS.

2.3.3.4 DNA Staining

After washing with PBS, the discs were incubated with Hoechst in PBS for 30 min at RT protected from light, and then washed twice with PBS and mounted in 85% glycerol 3% n-propyl gallate (w/v) before imaging by fluorescence microscopy.

2.3.4 X Gal staining

Imaginal discs were dissected from third instars larvae and collected in PBS. The discs were placed in fixative solution for 20 min in RT. After fixation, the discs were washed with PBT three times for 10 min each, and once in prewarmed X-Gal staining solution (without X-Gal) for 10 min. Then they were incubated in prewarmed X-Gal staining solution at 37°C for 1h to overnight and washed once in PBS then mounted the on a slide in 85% glycerol 3% n-propyl gallate (w/v).

Fixative solution: 1% glutaraldyde in PBS

Staining solution: 100 ml 1x PBS
150 mM NaCl
1mM $MgCl_2$
3.1 mM $K_4\{Fe(II)(CN)_6\}x3\ H_20$
3.1 mM $K_3\{Fe(III)(CN)_6\}x3\ H_20$

For staining: 8µl X-Gal stock solution was added to 200µl staining solution.
X-Gal Stock Solution: 10% X-Gal in DMSO (dimethylformamide), it was stored at -20°C.

2.3.5 RNA in situ hybridisation on imaginal discs

2.3.5.1 Preparation of the Dig RNA labeled probe

The DNA template for transcription was cloned into an appropriate transcription vector that contains SP6 and/or T7 promoters. After linearization of the DNA at a suitable restriction site downstream of the cloned insert, the DNA was purified and concentrated with Zymo DNA purification Kit. 1µg from the prepared DNA was used for *in vitro* transcription to generate a large amount of Dig-UTP labelled RNA as described in Dig RNA labelling Kit

(Roche, Cat. No, 11 175 025 910). RNA was purified and concentrated with Zymo RNA purification Kit. 20µl hybridisation buffer was added to the purified RNA and stored at -20°C.

2.3.5.2 Preparation of imaginal discs for in situ hybridization

Imaginal discs were dissected from third instars larvae in PBS and collected in ice cold PBS. The dissected material was placed in fixative solution for 20-30 min at RT. After fixation the discs were washed with PBT five times for 5 min each and were digested for 2 min at 55°C with Proteinase K (20µg/ml) in PBT solution. Imaginal discs were washed several times in ice cold PBT then eight times for each 5 min at RT. After 20-30 min post fixation with fixative solution the discs were washed in PBT four times for each 5 min at RT.

Fixative solution:	4% paraformaldehyde in 1 x PBS
PBT:	0.3% Triton X-100
	1x PBS

2.3.5.3 Prehybridization and hybridization of the prepared imaginal discs

The prepared imaginal discs were then prehybridized by incubating them for 10 min in 1:1 PBT: hybridization solution at RT followed by other 10 min in hybridization solution at RT. The discs were transferred into fresh hybridization puffer and incubated at 55°C for 1hr. After prehybridization the discs were hybridized overnight at 55°C with the antisense DIG (digoxigenin) labelled RNA probe that was diluted 1:10 in hybridization buffer and denatured for 10 min at 80°C (500ng/ml in hybridization buffer). The probe was removed and the discs were washed with hybridization solution for 20 min at 55°C, with a 1:1 mixture of hybridization solution and PBT at 55°C and then with PBT alone five times for each 20 min.

Hybridisation puffer:	50% formamide 5X SSC (20X 3M NaCl, 0,3M sodium citrate pH: 7,2) 100µg /ml *E.coli* tRNA 50µg/ml Heparin 0,1% Tween-20 pH: 4,5

2.3.5.4 Detection of in situ signal

The discs were incubated with the alkaline phosphatase conjugated goat anti-digoxigenin, (1:2000 in PBT) for at the least 2 hr at RT. Thereafter the discs were washed in PBT four

times for each 20 min, then in staining buffer tree times for each 5 min followed by the incubation in staining buffer containing 4.5 µl of 4 nitro blue tetrazolium chloride (NBT) and 3.5 µl 5-bromo-4-chloro-3-indoly-phosphat (BICP) per ml of solution. The reaction was stopped by several washes in PBS after the staining was visible. The discs were then transferred to 50% glycerol in PBS for 30 min and then mounted in 100% glycerol.

Staining buffer: 100 mM NaCl
50 mM $MgCl_2$
0.1% (v/v) Tween-20
100 mM Tris pH 9.5

2.4 Culture and manipulation of S2 cells

Cultured *Drosophila* cell lines have become an increasingly popular model system for cell biological and functional genomics studies. One of the most commonly used lines, *Drosophila* Schneider cells (S2 cells) (Schneider, 1972). S2 cells were originally derived from the late embryonic stage (20-24 hours old). These cells exhibit mesodermal characteristics and their repertoire of cellular behaviors and gene expression suggest that they are derived from hemocytes- professional macrophages that, in the fly, are responsible for phagocytosis of invading bacteria and apoptotic cells. Morphologically, S2 cell line is roughly spherical, ~ 15-20 µm in diameter, and adheres rather weakly to most tissue culture substrate (Rogers & Rogers, 2008). It is particularly useful as it is easy to grow and maintain in the lab. They divide rapidly, with about a 20-h cell cycle at room temperature (20-25ºC), and do not require specialized equipment for their culture apart from sterile hood for passage. Cultured *Drosophila* cell lines have become well-known experimental systems to use in combination with RNAi, as they are highly susceptible to RNAi after treatment with large double-stranded RNAs. In addition, the *Drosophila* genome exhibits less functional redundancy compared with mammalian systems, which makes it easier to produce a loss-of-function phenotype by inhibiting a single gene at a time. Finally, with the development of commercially available dsRNA libraries and screening facilities, fly cell lines have been used as gene discovery tools for a variety of cellular processes (Worby et al., 2001; Kao& Megraw, 2004; Schepers., 2004; Worby & Dixon., 2004; Rogers & Rogers, 2008;). Such as studding of cell cycle (Boutros et al., 2004; Eggert et al.,2004; Björklund et al., 2006; Lents& Baldassare, 2006; Bettencourt-Dias & Goshima 2009).

2.4.1 Culturing S2 cells

S2 cells are ideally kept in a clean 25-27ºC incubator. There is no need for controlled CO_2 atmosphere because the cells are tolerant to wide changes of pH. S2 cells grow in Schneider's-*Drosophila* Medium as a loose, semi-adherent monolayer in tissue culture flasks. When culturing the cells, an aseptic technique was used by wiping everything with 70%ethanol and working inside the tissue culture hood.

Schneider's-*Drosophila* Medium 500 ml stock solution (GIBCO)
+ 10% FCS Fetal Calf Serum
+ 600µl 1000x Penicillin-Streptomycin stock solution
} 1x solution stored at 4°C

2.4.1.1 Initiating cell culture from frozen stocks

The vial of frozen S2 cells supplied contains 1×10^7 cells. Upon thawing in (in 37 º C water bath), the cells were centrifuged for 3 min at room temperature and 900 rpm. The medium was removed with a sterile pipette and 1ml of 24ºC prewarmed Schneider's medium was added. The cells were resuspended many times and transferred in 25cm^2 cell culture flasks, and then 3ml fresh Schneider's-*Drosophila* medium was added. Cells were incubated at 28 ºC until they reached a density of 6-20 $\times10^6$ cells/ml. This may take 1-2 weeks.

2.4.1.2 Passaging the S2 Cells

Drosophila S2 cells double approximately every 24h. As such, they should be routinely split once to twice a week. For this, cells were resuspended from the bottom of the flask in their own media by pipetting. S2 cells should be split to a final density of 2 to 4 $\times10^6$ cells /ml in serum-containing medium. Depending on their density, cells were split at a 1:2 to 1:5 dilutions into new culture flasks. Complete Schneider's *Drosophila* medium was added up to 20 ml in 75cm^2 or 6ml in 25cm^2 flasks.

2.4.2 Cell titer determination

A cover glass was centered over the hemocytometer chambers. Cells were resuspended from the bottom of the flask in their own media by pipetting up and down. One chamber was filled with10 µl cell suspension and the solution was allowed to enter the chamber by capillary action. Under the microscope, the cells located in each of the four corners of the hemocytometer were counted, since this produces the best visualization of the cells. For counting, a hand-held counter

was used to record the number of cells counted. The number of cells/ml and the total number of cells were determined using the following formula: cells/ml = number of cells counted x 10^4

2.4.3 Vital staining with Trypan blue

This is a commercially method used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as Trypan blue, whereas dead cells do not. 10µl of 0,4% Trypan blue and 10µl of cell suspension were mixed and incubated about 3 min at RT. Cells should be counted within 3-5 min of mixing with Trypan blue, as longer incubation periods will lead to cell death and reduced viability counts. 10µl of the cell mixture was applied to a hemocytometer and the unstained (viable) and stained (nonviable) cells were counted separately. The percentage of viable cells was calculated as follows:
Viable cells (%) = total number of viable cells/total number of cells * 100
Trypan blue: was purchased from Serva.

2.4.4 Immunostaining of S2 cells

S2 cells were grown overnight on coverslips coated with poly-L-lysine in a 6-well plate, washed 2×10 min with PBSD and fixed for 10 min with 3,7% formaldehyde in PBSD, then washed 2×10 min with PBSD. The cells were then further fixed and permeabilized using the following solutions:
10 min in 50% Methanol, then 10 min in 100 % Methanol, 10 min in 50% acetone, and 10 min in100 % acetone all at -20ºC. After that, cells were washed for 10 min with PBSD, and blocked 2×10 min with buffer A. Then cells were immunostained with appropriately diluted primary antibody for 1 h at room temperature in a humid chamber. After washing, coverslips were incubated with a suitable labeled secondary antibody for 2h at room temperature in a humid chamber, and then washed and stained with Hoechst for 10 min to visualize DNA.

Poly-L-Lysine stock solution: 50mg Poly-L-Lysine /50ml H_2O. Stored at -20°C.
Poly-L-Lysine work solution: 50µg /ml

10x PBSD : 1.5M NaCl
100mM KH_2PO_4

10x Puffer A: 150 mM Tris-HCl, pH 7,5
600 mM KCl
150 mM NaCl
5 mM spermidine
1.5 mM spermine

2.4.5 EdU incorporation

S2 cells were grown overnight on coverslips coated with poly-L-lysine in a 6-well plate. Then cells were incubated with 0.3ml of 40µM prewarmed EdU in Schneider's medium for 3 or 6 hours at RT. Cells were washed 2×10 min with PBSD and fixed for 10 min with 3.7% formaldehyde in PBSD, and then cells were washed 2×10 min with PBSD. The cells were then further fixed and permeabilized using the following solutions:

10 min in 50% Methanol, then 10 min in 100 % Methanol, 10 min in 50% acetone, and 10 min in100 % acetone all at -20 ºC . After that cells were washed for 10 min with PBSD, and then cells were incubated protected from light for 30 min at RT with 0.3 ml Click-iT reaction to detect the EdU. Then cells were washed and stained with Hoechst.

3 Results

3.1 The role of Bx42/SKIP in cell cycle control during *Drosophila* eye development

3.1.1 Expression of the dominant negative Bx42-SNW in late 3rd instar eye discs results in reduction of eye size

The power of *Drosophila* lies in genetic analysis. Classical genetic screens for late larval/ pupal lethal mutants identified genes involved in the different cell cycle phases. The *Drosophila* eye has been the basis of numerous genetic screens to study growth, proliferation and signal transduction pathways that directly or indirectly impinge on the cell cycle. The relatively uniform size of the eye allows one to screen for mutations in genes that affect-proliferation. Forward genetic techniques are used to alter the expression of a gene of interest, followed by a screen for modifiers of the resulting phenotype. A useful tool in this screening strategy is the use of the GAL4 two component system. The system permits to overexpress a UAS-transgene (effector element) in a pattern that corresponds to the expression of a transgenic Gal4 yeast transcription factor that is under the control of a given DNA regulatory element (driver element). There are many GAL4 driver lines available in the Drosophila community (Fly Base, Bloomington) expressing the Gal4 tissue- or developmental stage-specific. In addition, by modifying the breeding temperature of the flies (in a range between18-29°C) the strength of Gal4 expression can be modulated. Any transgene containing Gal4 binding sites in front of its promoter (upstream activating sequence, UAS) can be induced. The system therefore may be used to tissue specifically overexpress certain proteins, or, by inducing ds-hairpin RNA, to knockdown certain genes. By genetic crossing combinations of driver and effector elements can be generated according to the requirements of the planned analyses. In my experiments, I used this system for the expression of transgenes of interest in the eye. I first wanted to establish a fly strain with a constant Bx42-loss of function phenotype. Later this strain should be used to test genetically for Bx42 interacting genes that were expected to turn as modifiers of this phenotype.

To establish a Bx42 phenotype to test its role for eye development, the dominant negative *UAS-Bx42-SNW* allele was crossed with four different eye-specifically expressed GAL4 driver lines. These lines express the desired protein in the whole or in parts of the eye, and so the impact of the Bx42-SNW expression at different developmental stages of eye development could be investigated (see introduction).

The *UAS-Bx42-SNW* transgene that contains the middle region of Bx42 only (amino acids 177-438) (Figure 3-1) was provided by Dr. Negeri. In this construct, both the N-terminus and thus the Su (H) interaction domain, and the C-terminus, containing the transactivation domain and the Hairless interaction domain of Bx42, are deleted. The presence of the

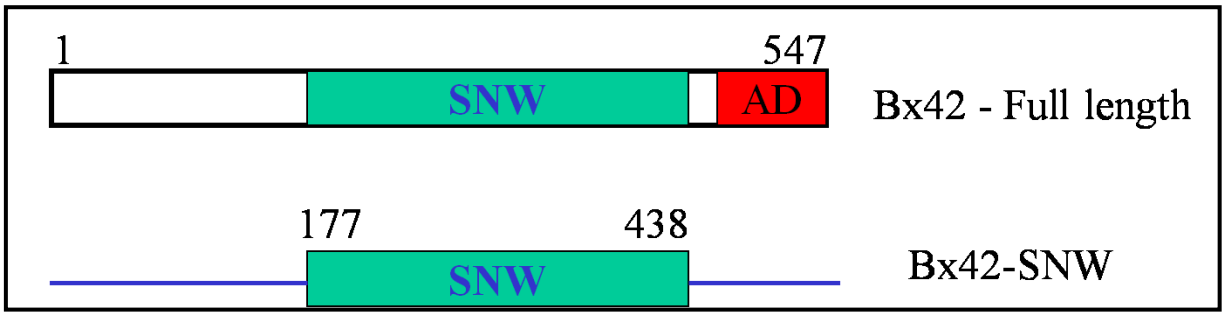


Figure 3-1: Schematic representation of Bx42 constructs.
Bx42- FL and Dominant negative Bx42-SNW. The number of amino acids for the N- terminus is indicated.

UAS-Bx42-SNW construct only as well as any of the GAL4- driver lines only did not result in any change of eye size or morphology (Figure 3-2 A, B).

GMR-GAL4 was reported to drive the expression of target genes in all cells posterior to the morphogenetic furrow in the developing eye (Song et al., 2000; Figure 3-3 A). A cross at 25°C between males, which are homozygous for a UAS-Bx42-SNW transgene on the third chromosome and homozygous females containing a second chromosomal *GMR*-GAL4 transgene generated progeny with small and rough eyes (Figure 3-2 C). Within the *GAL4/+; UAS-Bx42-SNW/+* progeny of this cross, the extent of eye size reduction is very similar and sex independent. When the full-length allele UAS-Bx42-FL was crossed with GMR-GAL4 no effect on eye development was observed (Figure 3-2 D), indicating that an increase in the Bx42 dosage does not disrupt eye development.

Drosophila atonal (*ato*) is a proneural gene required for cell fate specification and induction of differentiation in the PNS and the secretory lineages. In wild-type eye imaginal discs, *ato* is expressed in a large swathe of cells ahead of and within the morphogenetic furrow to specify R8 precursor's cells (Figure 3-3 B). Using *ato*-GAL4 as a driver line (crossing principally similar as described for GMR-GAL4) overexpression of Bx42-SNW in the *atonal* pattern results in flies that have normal eyes (Figure 3-2 E). Since *atonal* at that stage is expressed at a relatively low level Bx42-SNW expression maybe not induced strong enough to antagonize the effect of abundant endogenous Bx42 (see discussion).

The *ey*-GAL4 driver line like the *eyeless* gene is expressed in the whole eye disc of *Drosophila* larvae (Kumar & Moses, 2001). Overexpression of *UAS-Bx42-SNW* in the eye anlagen using *ey*-GAL4 results in flies that has a variable small-eye phenotype or lack most of the eye at 25°C (Figure 3-2 F,G, H, I, J, K, L). This driver line was unsuitable since the strength of the expression was variable among individual flies of the same cross. Variable expression induced by *ey*-GAL4 is demonstrated by the variable strength of UAS-GFP reporter expression in a separate experiment (Figure 3-3 D, E, F). At 29°C, the GAL4-UAS transactivator is more active and as a consequence in *ey*-GAL4; UAS-Bx42-

Figure 3-2: The effects of Bx42-SNW overexpression induced by different driver lines on eye development.
(A) Control: UAS-Bx42-SNW has wild type eyes, (B) Control: GMR-GAL4 has wild type eyes. (C) GMR-GAL4, UAS-Bx42-SNW has small and rough eyes. (D) GMR-GAL4/UAS-Bx42-FL has wild type eyes. (E) ato-GAL4/UAS-Bx42-SNW has wild type eyes. (F - L) ey-GAL4/UAS-Bx42-SNW individuals of the same cross showing variable small eye phenotype from slightly reduced to fully absent eyes. All were grown at 25C°.

SNW animals a fully penetrant eye loss was induced. Moreover, induction at this temperature is accompanied by pupal lethality, because flies with small heads or headless flies develop, which do not enclose the pupal case (data not shown). All in all this genetic background was unsuitable for further analysis.

The fourth eye driver line used was *elav*-GAL4. *elav* is required for correct differentiation and maintenance of the nervous system and it is expressed in the differentiating photoreceptor neurons posterior to the furrow of the eye imaginal disc (Figure 3-3 C). However, a cross between Bx42-SNW with elav-GAL4 was embryonic lethal (not shown) and therefore this driver line was not further used.

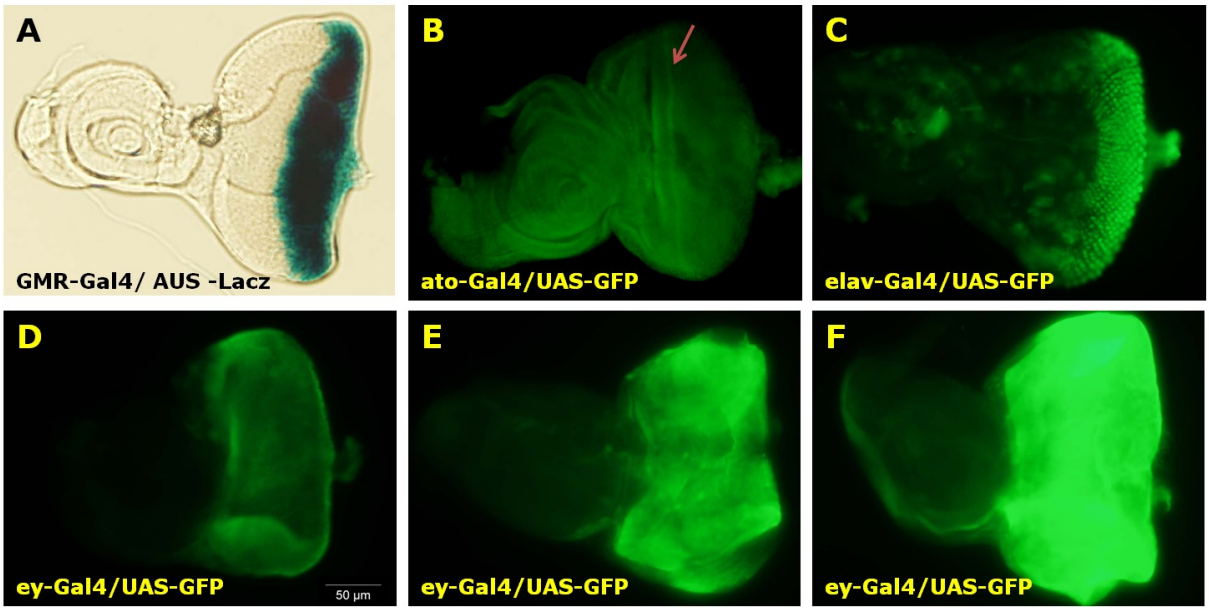


Figure 3-3: Expression of driver lines used as seen by LacZ- and GFP-reporter gene expression in eye discs.
(A) GMR-GAL4/UAS-lacZ eye imaginal disc stained with X-Gal, all cells posterior to MF were stained, (B) ato-GAL4/UAS GFP eye imaginal disc, GFP is expressed in R8 photoreceptor cells, posterior to MF (red arrow). (H) elav-GAL4/UAS GFP eye imaginal disc, GFP is expressed in all photoreceptor, posterior to MF. (D - F) ey-GAL4/UAS GFP eye imaginal discs, GFP is expressed variably in the eye disc. In this and all following figures anterior is to left. Bar 50µm.

3.1.2 Bx42-SNW induced reduction in eye size corresponds with a block of the cell cycle at the G2/M transition.

The striking reduction in eye size caused by the overexpression of Bx42-SNW could be a consequence of inhibition of proliferation and/or induction of cell death. In order to provide evidence for either of these assumptions, I examined the third instar eye imaginal discs for defects in cell proliferation and for induction of cell death specific genes. Only

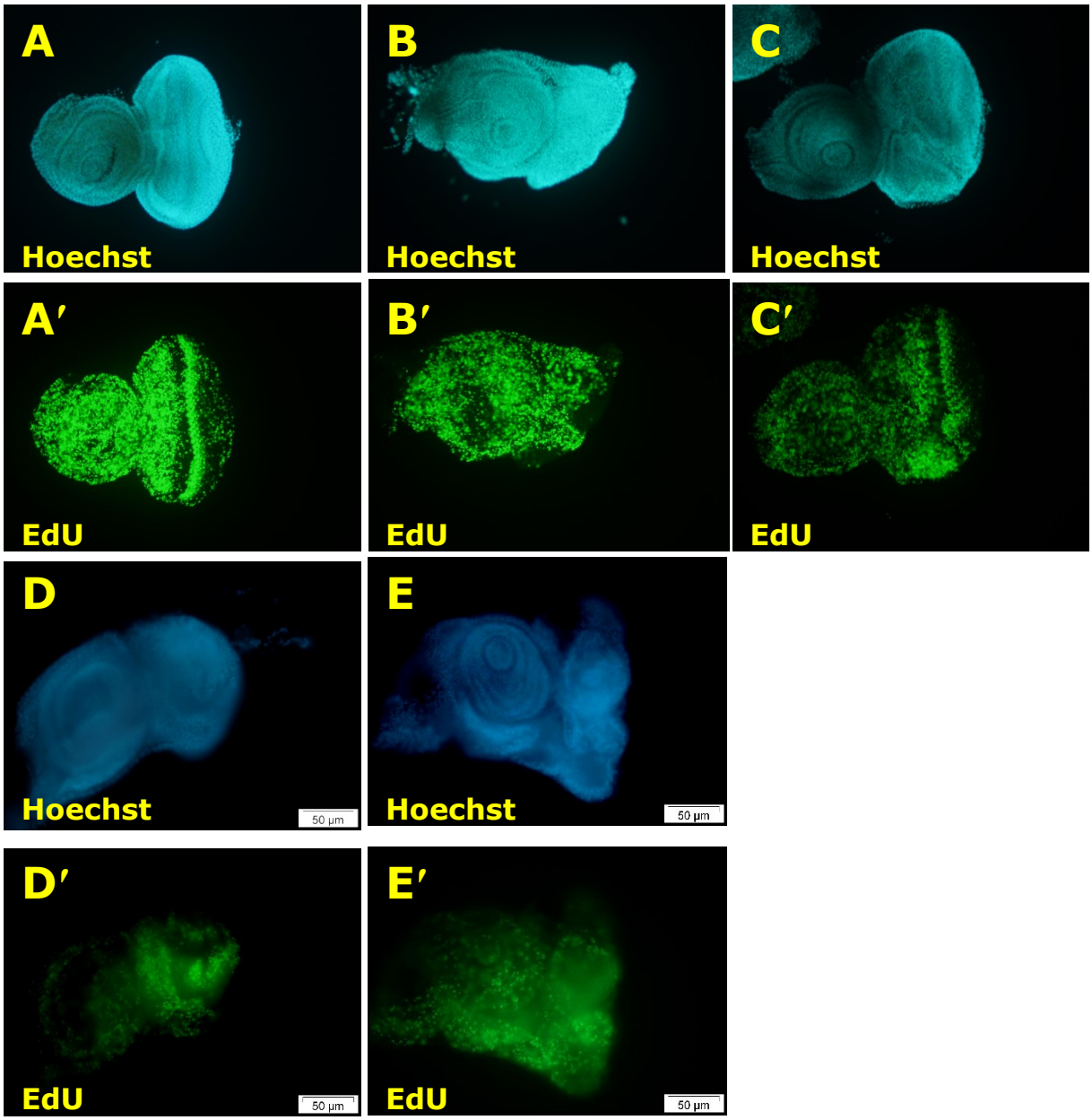


Figure 3-4: Eye driver lines EdU incorporation patterns.
(A, A′) ato-GAL4/UAS-Bx2-SNW eye imaginal disc, Hoechst staining (A) and EdU incorporation (A′) as WT. (B-E) ey-GAL4/Bx42-SNW eye imaginal discs show variable aberrant morphology in Hoechst staining and (B′-E′) variant disrupted EdU incorporation patterns. Bar 50µm.

the GMR-GAL4/ UAS-Bx42-SNW showed a significant and reproducible phenotype. Other driver lines were unsuitable for further analysis since they were embryonic lethal (*elav*-GAL4/ UAS-Bx42-SNW), didn't affect eye development (*ato*-GAL4/ UAS-Bx42-SNW; see fig. 3-2 E and fig 3-4, A, A´) or showed variant phenotypes in the adult eyes (*ey*-GAL4/ UAS-Bx42-SNW; see fig 3-2 F-L). Similar to the eye phenotype observed in adult flies *ey*-GAL4/ UAS-Bx42-SNW also revealed variant eye-disc phenotypes following Hoechst staining (Figure **3-4** B-E) and EdU incorporation (Figure **3-4**B´-E´). Some discs had a majority of cells in S-phase. Some discs showed abnormal morphology of the morphogenetic furrow, and aberrant EdU incorporation patterns. Therefore, the following experiments were done using GMR-GAL4/UAS Bx42-SNW.

3.1.2.1 Replication labelling of eye discs expressing Bx42/SNW under the control of GMR-GAL4 is indistinguishable from controls

Eye discs can be used to determine if there is an G1/S phase block by estimating the number of S-phase nuclei of Bx42-SNW animales in comparison to controls. In the wild type, random proliferation is observed in the anterior part and field of undetermined cells anterior to the morphogenetic furrow. Within the morphogenetic furrow, cells are synchronized to undergo a second wave of proliferation just behind the morphogenetic furrow. Once the cells differentiate they cease proliferation. Therefore EdU incorporation experiments of isolated eye imaginal discs were performed to label dividing cells that are in majority the S-phase of the cell cycle. Additionally, some cells could become labelled during the 90 min labeling period that were at the end of S- phase and would have enterered the following G2 phase. As documented in Figure 3-5, the pattern of replication

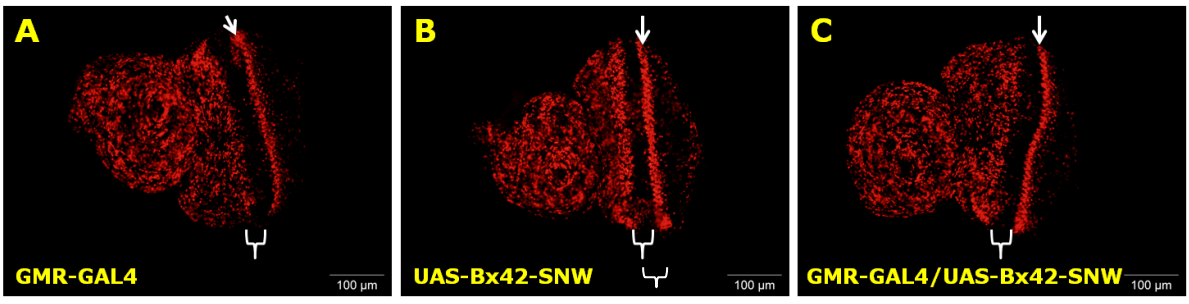


Figure 3-5: GMR-GAL4/UAS-Bx42-SNW eye imaginal discs showed no obvious change in the number of S phase cells in the second mitotic wave.
S phase cells in eye imaginal discs, labeled with EdU, of control (A) GMR-GAL4 (B) UAS-Bx42-SNW and (C) GMR-GAL4/UAS-Bx42-SNW third instar larvae eye imaginal discs. Arrow: second mitotic wave (SMW), Brace: morphogenetic furrow (MF). Bar 100µm.

labelling of GMR-GAL4/UAS-Bx42-SNW eye discs is very similar to that of GMR-GAL4 and UAS-Bx42-SNW controls as well as to wild type discs (not shown). However, slight differences might have been overlooked since a systematic cell count has not be performed.

3.1.2.2 The number of M-phase cells within the second mitotic wave region (SMW) is reduced in GMR-GAL4/UAS-Bx42-SNW animals

To further investigate cell cycle progression during M-phase, mitosis was visualized by immunostaining for phospho-histone H3 serine 10 (PH3). In controls expressing GMR-GAL4 alone or carrying the transgenic UAS-Bx42-SNW only, mitotic H3S10- staining was observed in the anterior disc and in few cell rows immediately posterior to the

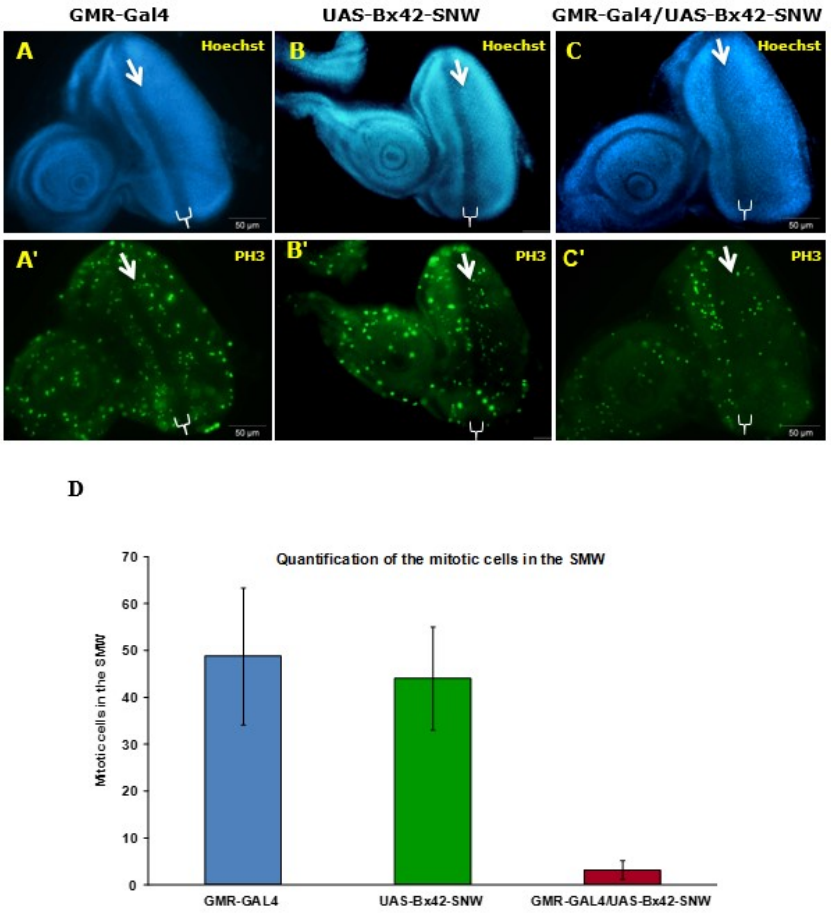


Figure 3-6: The proportion of cells in M -phase is reduced in the second mitotic wave (SMW) after Bx42-SNW overexpression.
Immunolabeling with anti-phospho histone H3 antibody marks cells in M phase. (A, A′) Control GMR-GAL4 and (B, B′) UAS-Bx42-SNW in the SMW show comparable number of M-phase cells following anti PH3 staining. (C, C′) the number of mitotic cells in the SMW is highly reduced in GMR-GAL4/UAS-Bx42-SNW cells. Arrow: second mitotic wave (SMW), Brace: morphogenetic furrow (MF). Bar 50µm. (D) Quantification of Mitotic cells in the SMW indicates a reduction in the number of the mitotic cells in GMR-GAL4/UAS-Bx42-SNW compared to the controls GMR-GAL4 and UAS-Bx42-SNW, 10 imaginal discs of every genotype were quantified.

morphogenetic furrow (second mitotic wave SMW) (Figure 3-6 A,A′ and B,B′). In GMR4/UAS-Bx42-SNW eye discs, the proportion of cells that stained with the anti-pH 3 antibody in the SMW field was decreased by about 94% (GMR-Gal4: 49 ± 15 cells; UAS Bx42-SNW: 44 ± 11 cells; GMR-Gal4/ UAS Bx42-SNW 3 ± 2 cells. Figure 3-6 C, C′, D). These results indicate that Bx42-SNW expressing cells in the SMW are compromised in their ability to progress through the cell cycle. They can proceed through G1/S but not in G2/M. A similar block in mitosis of eye disc cells was reported following Notch RNAi (Baonza & Freeman., 2005).

3.1.2.3 Cell death in eye discs of GMR-GAL4/UAS-Bx42-SNW animals is increased

To check whether the reduced eye size of GMR-GAL4/UAS-Bx42-SNW animals was due to cell death, in addition to cell cycle defect, imaginal discs were analyzed with antibodies directed against activated Caspase-3, which is a hallmark of apoptosis (Yu et al.,2002). In control eye discs GMR-GAL4 and UAS-Bx42-SNW of third instar larvae, there is very little apoptosis observed (Figure 3-7 A, A′ and B, B′). However, expression of Bx42-SNW under control of the eye-specific GMR enhancer posterior to the MF causes significant caspase-3 activity posterior to the MF as shown in (Figure 3-7 C, C′). As an additional test, eye imaginal discs were stained with acridine orange. Acridine orange is a vital dye that binds specifically to the DNA of dying cells. Apoptosis is characterized by the condensation of the chromatin. The DNA of apoptotic cells is fragmented, and becomes visible in the form of condensed "apoptotic bodies". These can be recognized under the microscope by acridine orange staining as bright spots and serve as markers of programmed cell death (Bonini, 2000). I observed acridin orange staining of some cells in the posterior region of control discs (*GMR-GAL4* or *UAS-Bx42-SNW* Figure 3-7 D,E). However, a significantly higher number of acridine orange stained cells was observed in the same region of eye imaginal discs of the *GMR-GAL4/UAS-Bx42-SNW* genotype (Figure 3-7 F). These results were similar to observations made following overexpression of the Notch antagonist Hairless (Müller et al, 2005).

Quantification of the caspase 3-positive cells in the posterior region revealed a notable increase with apoptosis. Average number of caspase-3 positive cells was 15 ± 7 in GMR-Gal4, 15 ± 5 in UAS Bx42-SNW and 144 ± 34 in UAS Bx42-SNW discs (Figure 3-7 G).

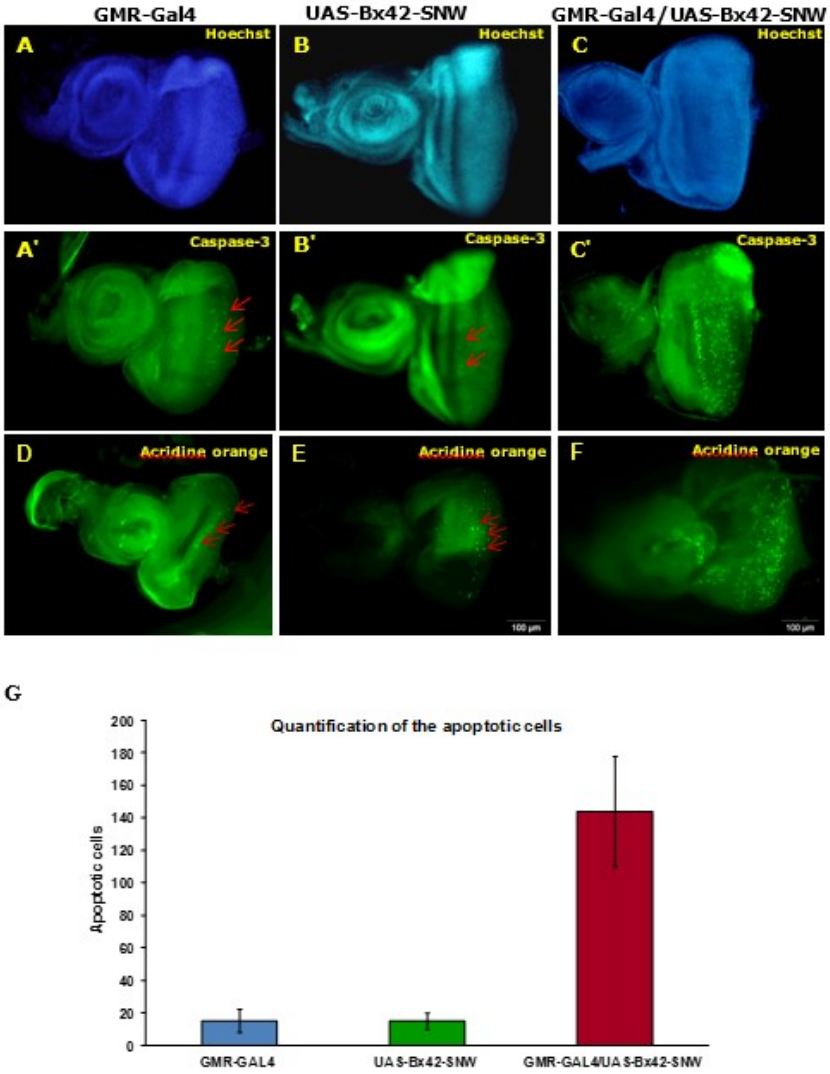


Figure 3-7: Bx42-SNW induces cell death in the eye imaginal disc.
Within the eye-antennal discs of controls: (A, A′) GMR-GAL4 and (B, B′) UAS-Bx42-SNW eye discs, there are only few apoptotic. Such cells detected by anti-cleaved Caspase-3 staining (green) are indicated with arrow. (C, C′) Overexpression of Bx42-SNW induces cell death and activation of caspase-3 in the region where Bx42-SNW seen by staining anti-cleaved Caspase-3 staining (green). Likewise, acridin orange staining only showed a few dying cells in (D) GMR-GAL4 and (E) UAS-Bx42-SNW. (F) Eelevated cell death was observed in GMR4, Bx42-SNW eye discs. (G) Quantification of the active caspase stained apoptotic cells indicates an elevation in cell death in the posterior part of the eye disc of GMR-GAL4/UAS-Bx42-SNW animals in comparison with the control GMR-GAL4 or UAS-Bx42-SNW eye discs.10 imaginal discs per line were quantified and are presented with the standard deviation of the mean. Bar 100µm.

3.1.3 Identification of genes involved in cell cycle regulation that modify the Bx42-SNW eye size phenotype.

To gain insight into the molecular mechanism of Bx42 involvement in the control of proliferation and to identify prospective mediators or regulators of this process, I designed genetic screens for modifiers of the dominant negative Bx42-SNW-mediated the small eye phenotype. As shown before flies expressing the GMR-GAL4/UAS-Bx42-SNW transgene combination have a small, rough phenotype. The intermediate phenotype caused by each one

copy of the GMR-GAL4 and one copy of the UAS-Bx42-SNW transgene makes these flies very useful for screening for modifiers (Figure 3-8 C). For example, flies' carrying two copies each of GMR-GAL4 and UAS-Bx42-SNW displayed a much more severe eye phenotype (Figure 3-8 B). I screened 70 available strains for modifiers of a small rough eye phenotype caused by overexpression the dominant negative Bx42-SNW. These lines include inducible RNAi transgenes and loss or gain of function alleles of genes that are involved in cell cycle regulation obtained from the Bloomington Stock Center and the Vienna *Drosophila* RNAi Centre (VDRC). Among the screened flies, eleven transgenic lines showed suppression or enhancement of the small rough eye phenotype caused by the UAS-Bx42-SNW (Appendix 1). These will be discussed below.

3.1.3.1 Modification of Bx42-SNW induced eye size phenotype by loss of function of cell cycle regulators.

My genetic screen revealed that the phenotype caused by Bx42-SNW is enhanced by a dominant negative form of EGFR. In *Drosophila* eye imaginal disc, R8 cells activate EGFR to arrest and recruit 4 other precluster cells (photoreceptor neuron R2-R5). Other cells lack EGFR activity, reenter the cell cycle and perform S phase. However, when these cells enter G2 phase, local control depends on short-range signals from R8 and R2–R5 cells activate EGFR to permit mitosis of these cells and promote G2/M progression during the SMW (Baker and Yu, 2001). Figure 3-8 D shows flies of the genotype GMR-GAL4, +/ +, UAS-EGFR DN; UAS-EGFR. DN/+ with one copy of the GMR driver transgene and two copies of a dominant negative form of EGFR. Flies with this genotype have small eyes, as was reported previously (Wang et al., 2008). This small eye phenotype is further enhanced by the addition of one copy of a UAS-Bx42-SNW transgene (Figure 3-8 E). This enhanced small eye phenotype in GMR-GAL4, +/ +, UAS- EGFR DN; UAS-EGFR, +/ +, UAS-BX42-SNW flies could be explained as an additive effect. Alternatively, it may be mediated by the negative effects of Bx42-SNW on Notch regulation, since Notch and EGFR signaling crosstalk has been often described (Tsuda et al., 2002; Firth & Baker., 2005; Müller *et al* .,2005; Doroquez & Rebay., 2006; Protzer et al., 2008; Krjci *et al* ., 2009).

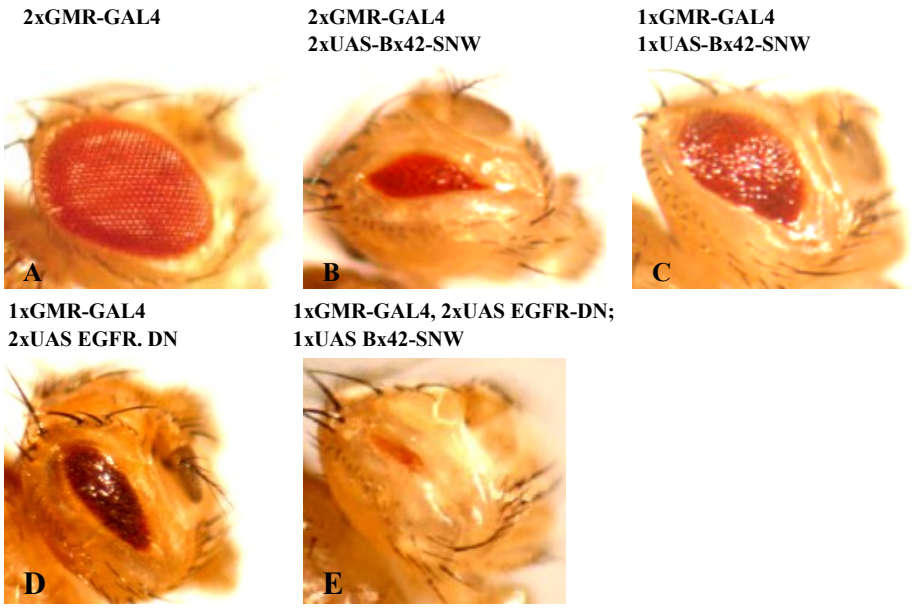


Figure 3-8: Enhancement of Bx42-SNW eye phenotype by expression of dominant negative EGFR:
(A) GMR-GAL4. Control eye phenotype. (B) GMR-GAL4/GMR-GAL4; UAS-Bx42-SNW/ UAS-Bx42-SNW. Enhanced small eye phenotype because of the presence of two copies of UAS-Bx42-SNW transgene, (C) GMR-GAL4/+; UAS-Bx42-SNW/+. Small eye phenotype caused by one copy of UAS-Bx42-NW transgene. (D) GMR-GAL4, +/+, UAS-EGFR-DN; UAS-EGFR-DN/+. Small eye phenotype caused by the presence of two copies of EGFR-DN transgene. (E) GMR-GAL4,+/ +, UAS-EGFR-DN; UAS-Bx42-SNW,+/+,UAS-EGFR-DN. Strongly enhanced small eye phenotype by two copies of EGFR-DN and one copy of UAS-Bx42-SNW.

The retinoblastoma gene was the first tumour suppressor gene to be identified. Its product, Rb, is a negative regulator of cell proliferation. Active Rb interacts with many nuclear proteins, including numerous transcription factors and chromatin-associated proteins (reviewed by Van den Heuvel and Dyson. 2008). Additionally, accumulating evidence indicates that Rb contributes not only to cell proliferation, but also to a diversity of cellular functions, including differentiation, cell death, senescence and genome stability (reviewed by Gordon & Du, 2011). As reported previously, Prathapam and colleagues (2002) found that the human homolog of Bx42, SKIP interacts with Rb and, in cooperation with Ski, can overcome Rb-induced transcriptional repression in human cells. My experiments revealed a genetic interaction between Bx42 and Rb, as I found that the Rb RNAi alone causes no change in the size of the eye (Figure 3-9 A-B). However, the small eye phenotype caused by Bx42-SNW was enhanced by simultaneous RNAi knock down of Rb (Figure 3-9 C-D). Similarly, the small eye phenotype is further enhanced by the addition of one copy of *Rb* loss of function allele (Figure 3-9E-F).

Tribbles is a cell cycle regulator that acts by specifically inducing degradation of the CDC25 mitotic activators String and Twine via the proteasome pathway. By regulating CDC25, Tribbles serves to coordinate entry into mitosis with morphogenesis and cell fate determination (Mata, 2000). Among the screened lines, a *tribbles* mutant line was found to

cause no change when expressed using GMR-GAL4 (Figure 3-10 A, B), but it suppresses slightly the Bx42-SNW small eye phenotype (Figure 3-10 C, D).

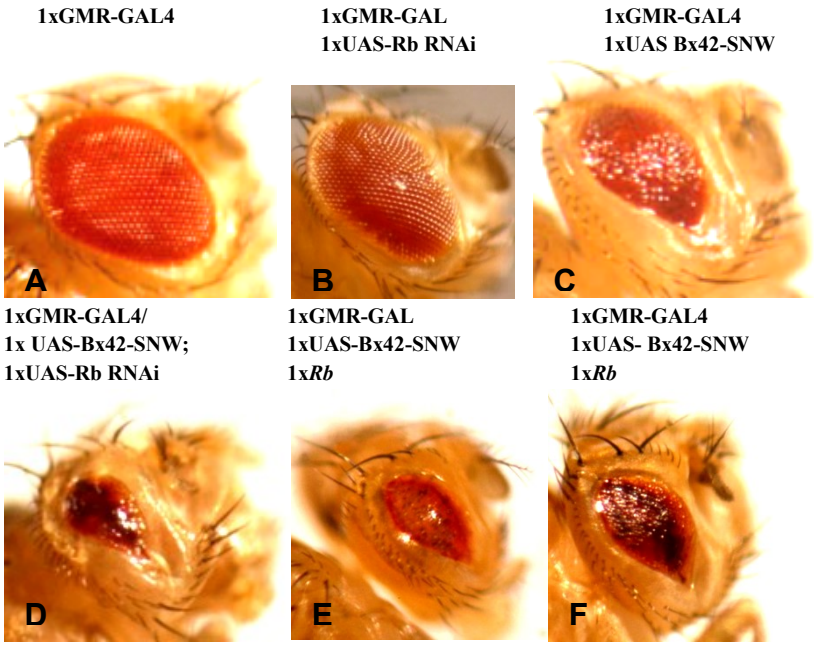


Figure 3-9: Enhancement of Bx42-SNW small eye phenotype by Rb RNAi.
(A) GMR-GAL4. Control eye phenotype. (B) GMR-GAL4/+; UAS Rb RNAi/+. Eye size is similar to controls (C) GMR-GAL4/+; UAS-Bx42-SNW/+. Small eye phenotype caused by one copy of Bx42-SNW transgene. (D) GMR-GAL4/+; UAS-Bx42-SNW, +/+, UAS-Rb RNAi. Enhanced small eye phenotype by one copy of Bx42-SNW and the presence of one copy of the UAS- Rb RNAi construct. (E, F) Rb/+; GMR-GAL4/+; UAS-Bx42-SNW/+. Enhanced small eye phenotype by one copy of Bx42-SNW and the presence of one copy of the *Rb* mutant (stock Nr: 7435 and stock Nr: 16803).

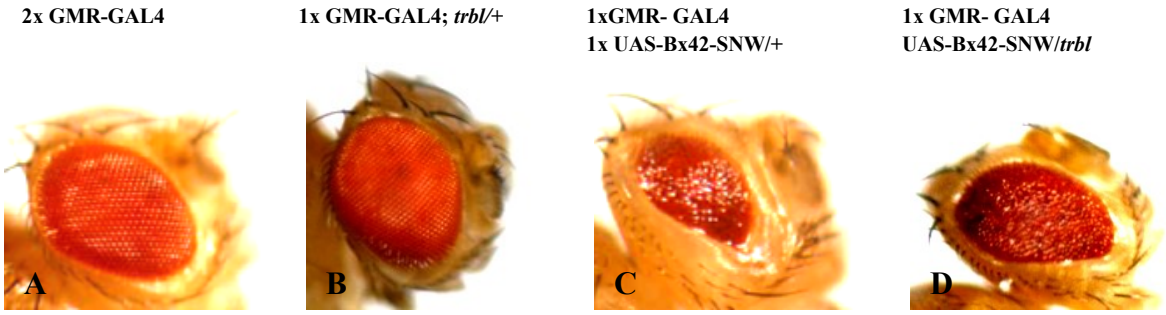


Figure 3-10: suppression of Bx42-SNW small eye phenotype by interaction with *tribbles*.
(A) GMR-GAL4. Control eye phenotype. (B) GMR-GAL4/+; *trbl*/+. Eye size is similar as in the controls. (C) GMR-GAL4/+, UAS-Bx42-SNW/+. Small eye phenotype caused by one copy of Bx42-SNW transgene. (D) GMR-GAL4/+; UAS-Bx42-SNW, +/+, *trbl*. *trbl* mutant allele suppresses slightly the Bx42-SNW induced eye size reduction.

3.1.3.2 Overexpression of genes involved in cell cycle regulation modifies the Bx42-SNW small eye phenotype.

In my screen, I identified three genes that on overexpression suppress the GMR-GAL4/UAS-Bx42-SNW eye phenotype. The first one is Cyclin B3, a cell cycle regulator involved in the regulation of G2/M transition and cytokinesis that promotes the metaphase-anaphase transition. Overexpression of UAS-Cyclin B3 using GMR-GAL4 driver line does not affect the eye size, but if simultaneously overexpressed with UAS-Bx42-SNW it suppressed the Bx42-SNW small eye phenotype though not the rough appearance (Figure 3-11 A, B, C, D). The second is the Cyclin dependent kinase Cdk1 (also known as Cdc2).

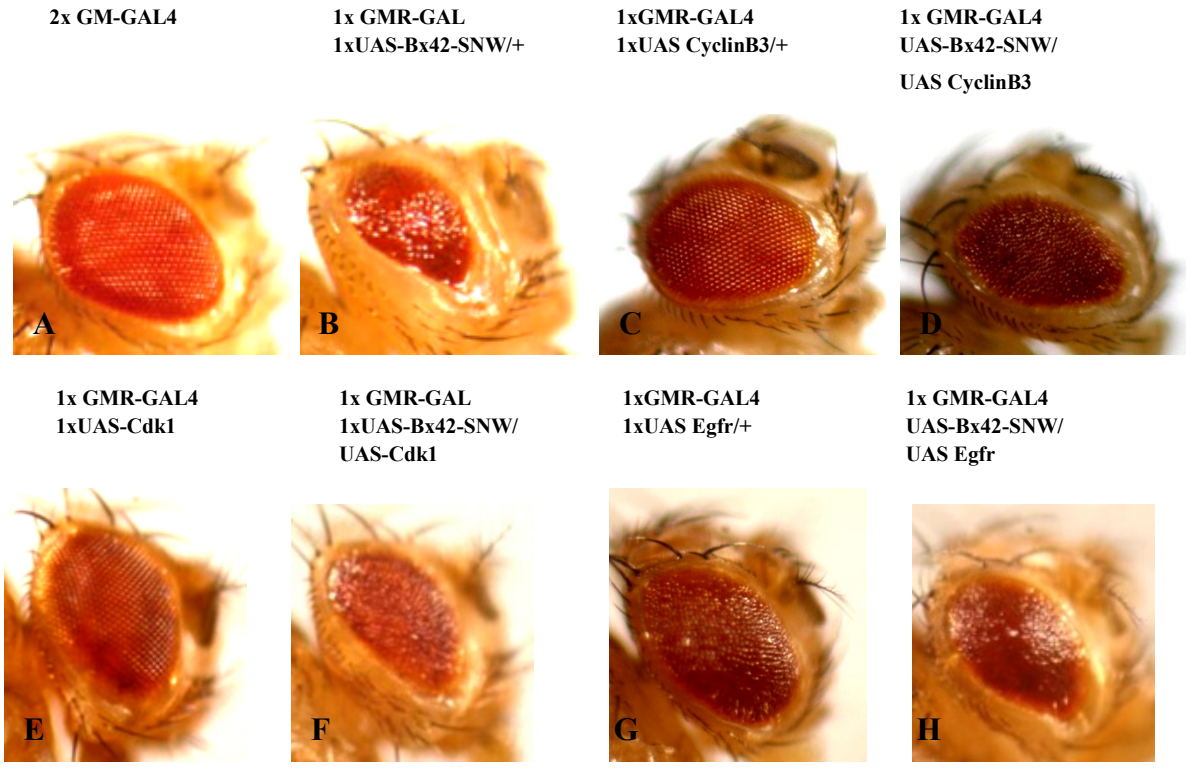


Figure 3-11: Overexpression of genes involved in cell cycle regulation suppresses the Bx42-SNW eye phenotype.
(A) GMR-GAL4. Control eye phenotype. (B) GMR-GAL4/+; UAS-Bx42-SNW/+. Small eye phenotype caused by one copy of Bx42-SNW transgene. (C) GMR-GAL4, +/+, UAS-Cyclin B3. Eye size is similar to controls. (D) GMR-GAL4, +/+, UAS-Cyclin B3; UAS-Bx42-SNW/+. Bx42-SNW small eye phenotype is suppressed in the presence of one copy of UAS-CyclinB3. (E) GMR-GAL4/+; UAS-Cdk1/+. Eye phenotype is like the control eye phenotype. (F) GMR-GAL4/+; UAS-Bx42-SNW,+/+,UAS-Cdk1. Bx42-SNW small eye phenotype is suppressed in the presence of one copy of UAS-Cdk1. (G) GMR-GAL4/+; UAS- EGFR/+. Rough eye phenotype but eye size is similar to controls. (H) GMR-GAL4/+; UAS-Bx42-SNW, +/+, UAS-EGFR. The small eye size phenotype induced by Bx42-SNW is slightly suppressed by presence one copy of UAS-EGFR transgene.

Cdk1 is the catalytic subunit of the Cyclin/Cdk heterodimer responsible for the execution of the M-phase of the cell cycle. As shown previously (Kanao et al., 2012), overexpression of Cdk1 using GMR-GAL4 resulted in WT eye size (Figure 3-11E). Interestingly, overexpression together with UAS-Bx42-SNW rescued the small eye phenotype of Bx42-SNW (Figure 3-11F).

Overexpression of EGFR with GMR-GAL4 reported previously (Hagedorn et al., 2006) causes a rough eye phenotype (Figure 3-11G). Activation of EGFR signaling via overexpression of the activated EGFR in a GMR-GAL4/+; UAS Bx42-SNW/+ background partially suppressed the Bx42-SNW small eye phenotype (Figure 3-11 H).

However, overexpression of genes involved in cell cycle regulation also may enhance the Bx42-SNW eye phenotype. The *Drosophila decapentaplegic (Dpp)* gene encoding a secreted protein of the transforming growth factor TGF-β super family, controls proliferation and patterning in diverse tissues, including the eye imaginal disc. Overexpression of Dpp using GMR-GAL4 resulted in eye phenotype similar to controls (Figure 3-12 C). However, expressing of Dpp by GMR-GAL4 in an UAS Bx42-SNW background further decreased the eye size and enhanced the Bx42-SNW eye phenotype (Figure 3-12 D). This is in accordance with data of Müller et al (2005) who found that activation of the Dpp pathway by overexpression either of the morphogen Dpp or of downstream components such as Dpp receptor Thick veins decreased eye size in GMR-GAL4/ UAS-Hairless genetic background with weakened Notch signaling due to enhanced Hairless expression.

E2F/DP is a heteromeric transcription factor containing a subunit encoded by a member of E2F family of genes and a subunit encoded by the *DP* gene. Flies carrying one copy of GMR-Dp and GMR-E2F showed only minor defects (Du et al, 1996). I could confirm this, since the overexpression of one copy of UAS-DP and UAS-E2F using GMR-GAL4 showed abnormal eyes but eye size was not changed (Figure 3-12 E). In contrast, animals induced by GMR-GAL4 which had one copy each of UAS DP, UAS E2F and UAS Bx42-SNW were pupal lethal. Although 92% of these flies died at the pupal stage, the few escapers obtained have a strongly enhanced Bx42-SNW small eye phenotype (Figure 3-12 F).

Drosophila's Armadillo (arm) is a multifunctional protein implicated in both cell adhesion, as a catenin, and cell signaling, as part of the Wingless (wg) signal transduction pathway. Wg signaling causes nuclear translocation of Armadillo, which then complexes with the DNA-binding protein TCF, enabling it to activate transcription. Expression of *Wg* or activated Arm throughout the eye disc reduces eye size (Singh et al., 2006) in addition to blocking differentiation **(**Hazelett et al., 1998; Lee and Treisman., 2001). In agreement with the previous studies, I found that overexpression of Armadillo using GMR-GAL4 resulted in small eyes phenotype, and this small eye phenotype was further enhanced by simultaneous overexpression of Bx42-SNW (Figure 3-13).

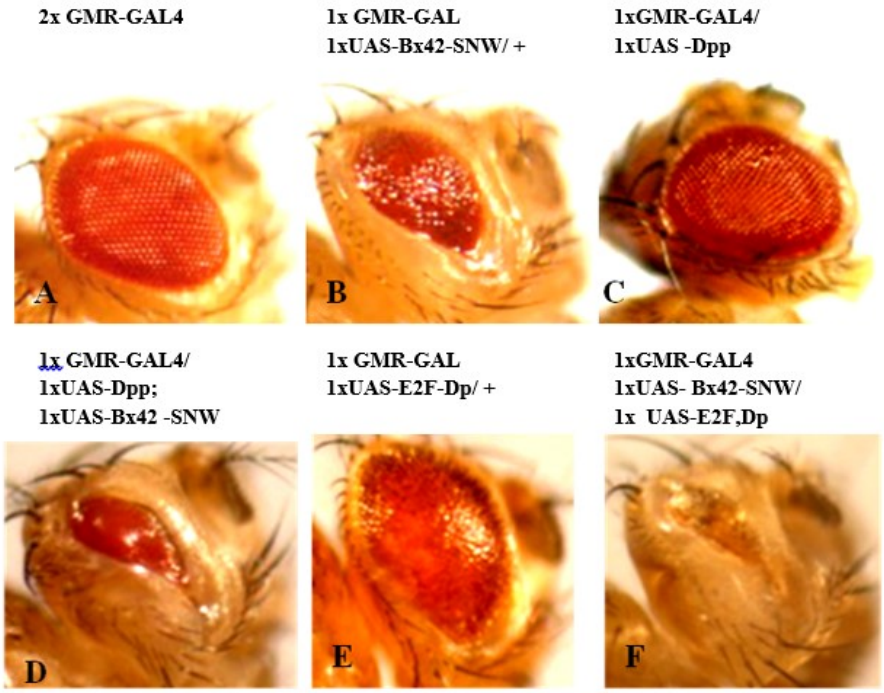


Figure 3-12: Overexpression of genes involved in cell cycle regulation enhances the Bx42-SNW eye phenotype.
(A) GMR-GAL4. Control eye phenotype. (B) GMR-GAL4/+; UAS-Bx42-SNW/+. Small eye phenotype caused by one copy of Bx42-SNW transgene. (C) GMR-GAL4, +/+, UAS-Dpp. Overexpression of Dpp by GMR-GAL4 resulted in eye phenotype similar to controls. (D) GMR-GAL4, +/+, UAS-Dpp; UAS-Bx42-SNW/+. Enhanced small eye phenotype caused by one copy of UAS-Bx42-SNW and one copy of UAS-Dpp transgene. (E) GMR-GAL4/ +; UAS-E2F, DP/+. Abnormal eyes with more bristles were apparent when UAS E2F, DP was overexpressed by GMR-GAL4. However, eye size was similar to controls. (F) GMR-GAL4/ +; UAS-Bx42-SNW,+ /+,UAS-E2F,Dp. Strongly enhanced small eye phenotype in escaper flies caused by one copy each of UAS-Bx42-SNW, UAS E2F and UAS Dp transgenes.

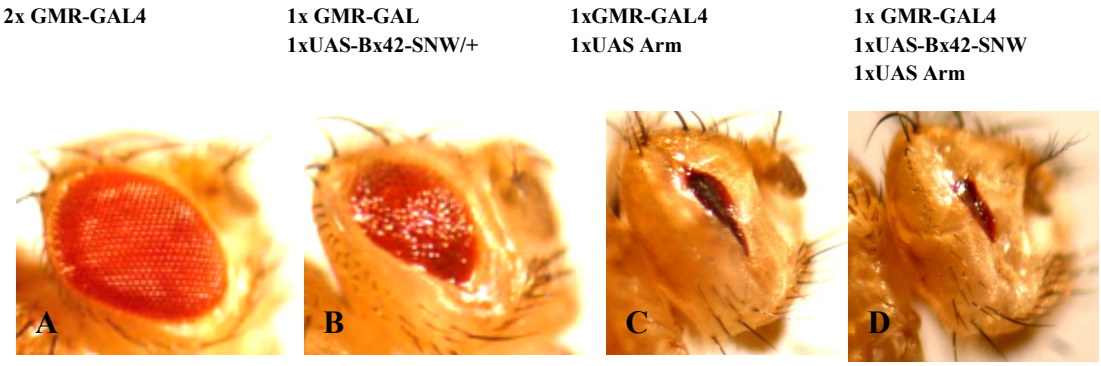


Figure 3-13: Overexpression of Armadillo enhances the Bx42-SNW eye phenotype.
(A) GMR-GAL4. Control eye phenotype. (B) GMR-GAL4/+; UAS-Bx42-SNW/+. Small eye phenotype caused by one copy of Bx42-SNW transgene. (C) UAS-Arm/ +, GMR-GAL4/+. Overexpression of Arm by GMR-GAL4 resulted in small eye phenotype.
(D) UAS-Arm/ +; GMR-GAL4/+; UAS-Bx42-SNW/+. Enhanced small eye phenotype caused by one copy of UAS-Bx42-SNW and one copy of UAS-Arm transgene.

3.1.4 Expression of genes involved in cell cycle regulation is reduced in eye discs of GMR4/ Bx42-SNW animals (Cyclin A, Cyclin B)

The cell cycle defects observed under conditions of overexpression of UAS-Bx42-SNW/GMR-GAL4 and the observed interactions with cell cycle regulatory genes prompted me to investigate whether the expression of cell cycle control genes was changed. Cyclin proteins act as regulatory subunits of protein kinase complexes involved in the control of eukaryotic cell cycle progression. In *Drosophila*, Cyclin E is necessary for the G1 to S phase transition while Cyclins A and B are necessary for progression from G2 into mitosis, although Cyclin A also plays a role in S phase (reviewed by Follette and O'Farrell, 1997). Cyc E protein in the developing eye imaginal disc of control GMR-GAL4 flies revealed by anti-CycE antibody staining showed essentially same distribution

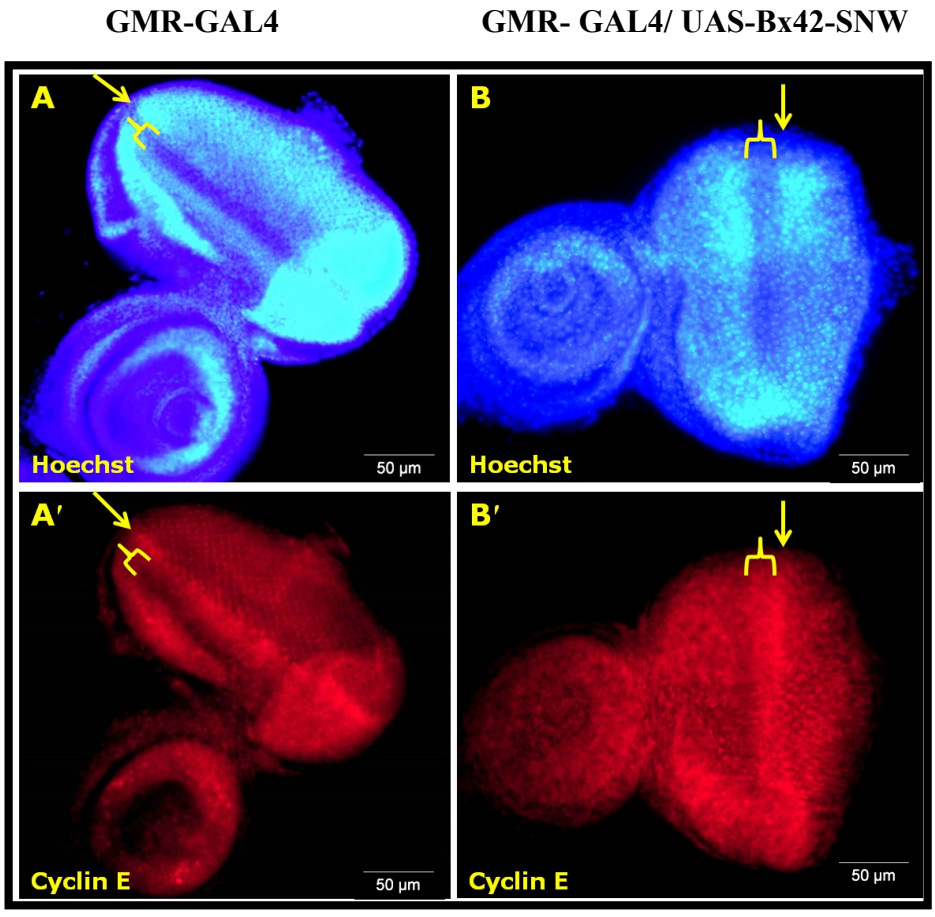


Figure 3-14: Overexpression of Bx42-SNW does not affect Cyclin E protein levels.
(A, B) Hoechst staining of control GMR-GAL4 and GMR-GAL4; UAS-Bx42-SNW eye imaginal discs. (A′, B′) Cyc E protein distribution in the same discs. Cyclin E is present in asynchronously dividing cells anterior, and absent from the G1 cells anterior to the MF and present in 3-4 rows of the SMW in the control eye discs. Cyc E protein distribution in the developing eye disc of GMR-GAL4; UAS-Bx42-SNW resemble the distribution of control. Arrow: second mitotic wave (SMW), Brace: morphogenetic furrow (MF). Bar 50µm.

as reported for WT flies by Richardson and coworkers (1995), as it is present in a subset of the asynchronously proliferating cells and in a band of cells immediately posterior to the MF (Figure 3-14 A,A′). Consistent with the results of EdU incorporation, Cyclin E expression wasn't reduced in Bx42-SNW expressing eye discs comparable to control eye discs, Although the cell arrangement and the Cyclin E activity looks much less ordered in the Bx42-SNW disc (Figure 3-14 B, B′), this unordered pattern distribution may be because Bx42-SNW disrupts the ommatidial patterning (Negeri unpublished data).

The G2-M Cyclins, Cyclin A and Cyclin B, are expressed posterior to the MF in a domain that is complementary and slightly posterior to cells with high levels of Cyclin E. To examine if the expression of Cyclin A was changed in Bx42-SNW, Cyclin A protein distribution was detected using anti- Cyclin A antibody. In the anterior region of the eye disc of control GMR-GAL4 flies, cells expressing Cyclin A were randomly distributed, reflecting the asynchronous mode of these cell cycles, Cyclin A was not detected within

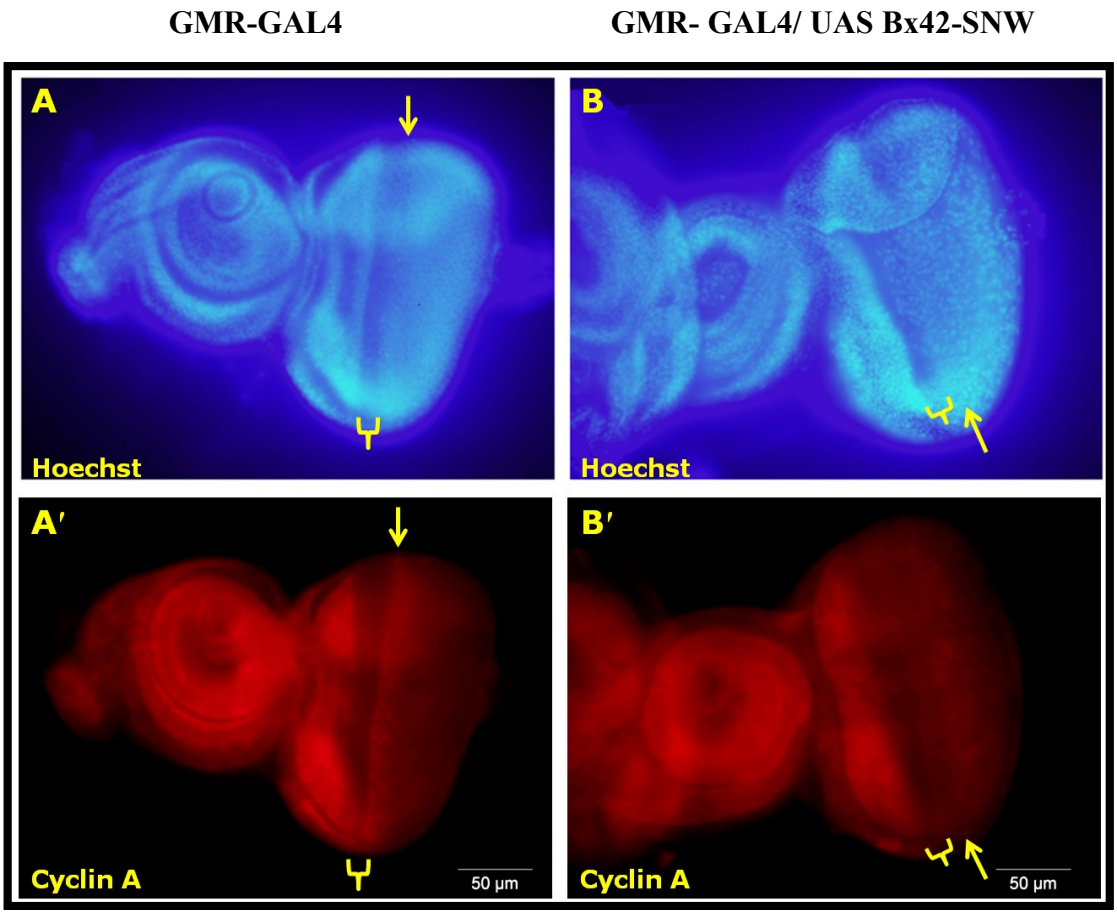


Figure 3-15: Reduced Cyclin A expression in the SMW of the GMR-GAL4/UAS-Bx42-SNW eye imaginal disc.
(A, B) Eye imaginal discs from GMR-GAL4 control and GMR-GAL4; UAS-Bx42-SNW animals respectively stained with Hoechst, (A′, B′) and immunostained with anti-Cyclin A antibodies showed the distribution of Cyclin A protein anterior to MF, and in a stripe of cells posterior to it (in the SMW) in the control, but not in the SMW of GMR-GAL4; UAS-Bx42-SNW flies. Arrow: second mitotic wave (SMW), Brace: morphogenetic furrow (MF). Bar 50µm.

the MF, consistent with these cells being arrested in G1. The G2-M cells posterior to the MF expressed Cyclin A in a domain that is complementary and slightly posterior to cells with high levels of Cyclin E (Figure 3-15 A, A′). In comparison to the control, Cyclin A was strongly diminished in the SMW region of GMR-GAL4/ UAS-Bx42-SNW eye imaginal discs (Figure 3-15 B, B′). Similarly to Cyclin A, Cyclin B is expressed randomly in the anterior region of the eye disc and in the SMW of the control GMR-GAL4 flies (Figure 3-16 A, A′). Compared to the control eye disc, Cyclin B signal in the region of the SMW was significantly reduced when Bx42-SNW was expressed in the eye disc (Figure 3-16 B, B′). Insufficient levels of the mitotic Cyclins (Cyclin A and Cyclin B) at the SMW, as shown above, may provide an explanation for the observed G2/M-specific cell cycle defects. (see 3.1.2.2).

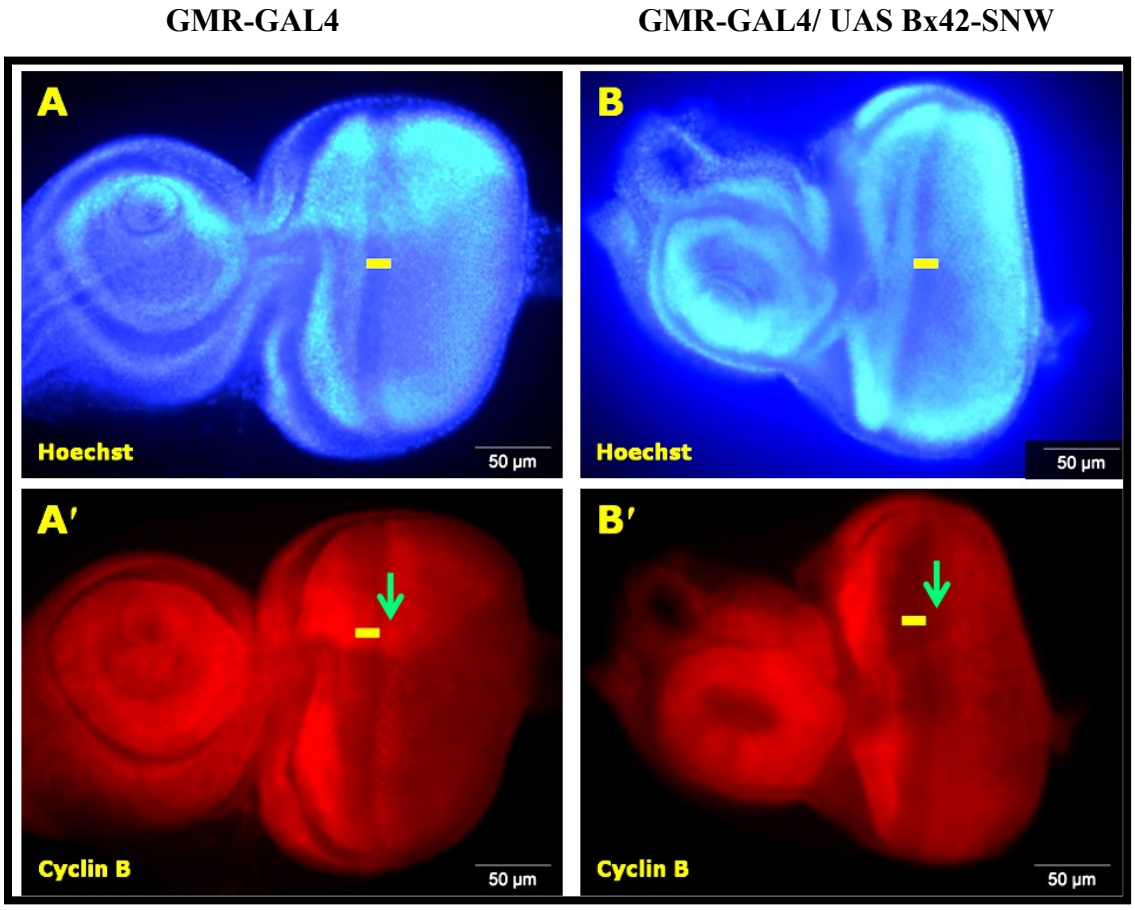


Figure 3-16: Reduced Cyclin B expression in the SMW in GMR-GAL4/UAS-Bx42-SNW eye imaginal disc.
(A, B) Eye imaginal discs from control GMR-GAL4 and GMR-GAL4; UAS-Bx42-SNW respectively stained with Hoechst. (A′, B′) Cyclin B immunostaining of control GMR-GAL4 and GMR-GAL4; UAS-Bx42-SNW respectively. Cyclin B staining showed WT pattern of cell cycle progression in GMR-GAL4. Cells enter S-phase of the SMW after unlocking G1 arrest in the MF, and most cells perform mitosis and degrade their Cyclin B between columns 3–5 of the SMW. Bx42-SNW prevents entry into the SMW in GMR-GAL4/UAS- Bx42-SNW flies, so that no cells contain Cyclin B in the SMW region posterior to the morphogenetic furrow. Arrow: second mitotic wave (SMW), Brace: morphogenetic furrow (MF). Bar 50µm.

Since Cyclin E functions through regulating the activity of its partner Cdk2, and since Cyclin E/Cdk2 kinase activity can be inhibited by Dacapo (Dap), a homolog of the p21/p27/p57 family of Cdk inhibitors, the protein level of Cyclin E may not always reflect the activity of Cyclin E/Cdk2 kinases. Actually, overexpression of Dap, which inhibits Cyclin E-dependent kinase activity, at the same time induced Cyclin E expression and protein accumulation (Lane et al., 1996; Reis & Edgar., 2004). To determine any effects of removing Bx42 on Dap expression, I have examined the expression pattern of Dap in GMR-GAL4, UAS Bx42-SNW eye imaginal discs at the RNA level using in situ hybridization. As shown in (Figure 3-17 A, B), no difference in the expression pattern of Dap could be found between the control and GMR-GAL4, UAS Bx42-SNW discs.

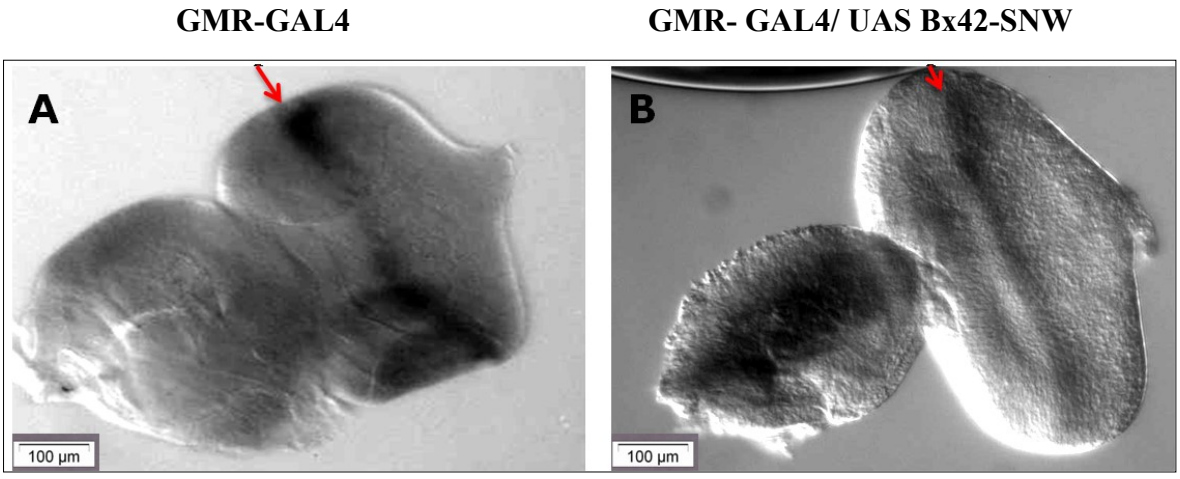


Figure 3-17: Dap expression was not affected by Bx42-SNW overexpression.
(A, B) Expression of *dap* was detected by RNA in situ hybridization in eye imaginal discs from control GMR-GAL4 and GMR-GAL4; UAS-Bx42-SNW respectively, (C, D). As shown, no differences in dap expression could be detected. Arrow indicates the cells expressed Dacapo, Bar 100µm.

3.2 Investigating the role of SKIP/Bx42 *Drosophila* S2 cells

The experiments in the first part of my thesis suggested a role for Bx42 in control of proliferation and cell survival. This should now be investigated in more details in a systematic cell culture approach using RNAi knockdown. Cultured cells provide a convenient source of homogeneous material of a rather defined origin. *Drosophila* S2 cells are suitable for inducing RNAi knockdown and furthermore, they are of a different stage of differentiation and tissue origin then the eye imaginal discs. Therefore, to complement the results of the first section of my thesis they were selected for these experiments.

3.2.1 RNAi knockdown of Bx42 in S2 cells results in arrest of cell proliferation

3.2.1.1 RNAi using Bx42 dsRNA results in cell cycle arrest

To study the role of Bx42 in *Drosophila* Schneider cells S2, I first analysed the consequences of depleting Bx42 by RNAi. The experiments were carried out with RNAi against two non-overlapping regions of the Bx42-coding sequence (Figure 3-18 A)

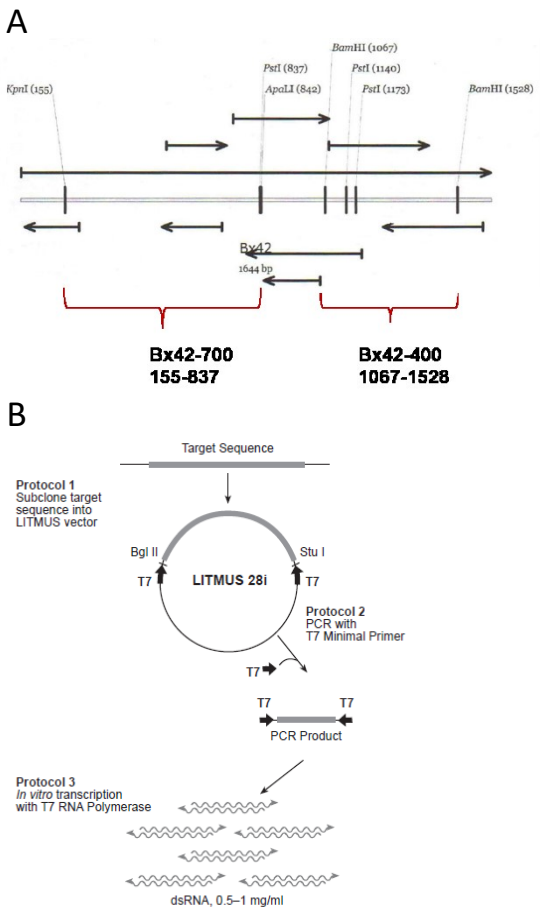


Figure 3-18: Bx42RNA interference.
(A) The regions of Bx42 gene used for RNAi. (B) Production of dsRNA from an insert cloned in LITMUS 28i. The target sequence is cloned firstly into of the LITMUS vector. Then, the insert is amplified by PCR with the supplied T7 Primer, producing a linear product with the target sequence flanked by T7 promoters. Double-stranded RNA is produced in high yield directly from PCR product.

Obtained using the database from DRSC & TRiP *Drosophila* RNAi Screening Centre, (www.flyrnai.org). One fragment is about 400 bp (Bx42-400) the other is 700 bp long (Bx42-700). As a control, RNAi against Orange fluorescent protein (OFP) that does not exist in *Drosophila* Schneider cells was used to show that dsRNA transfection in S2 cells affects *Drosophila* genes only. The target sequence was first cloned in LITMUS 28i vector (Logical

in vitro Transcription, Multiple Unique Sites), a vector that features a flexible polylinkers flanked by T7 promoters in opposite orientation. For in vitro transcription, the cloned insert was amplified by PCR using a single T7 promoter-specific primer, generating a double-stranded template whose ends are defined by the T7 promoters. Thus, in vitro transcription of the PCR product will produce double-stranded RNA without the need for separate restriction digestions (Figure 3-18 B).

Next, I investigated the effects of knockdown of Bx42 on *Drosophila* S2 cell growth. Firstly, 8µg dsRNA Bx42-400, dsRNA Bx42-700 and dsRNA OFP as a control were transfected to S2 cells (10^6 cells/3ml), and the cell number was counted every 24h.

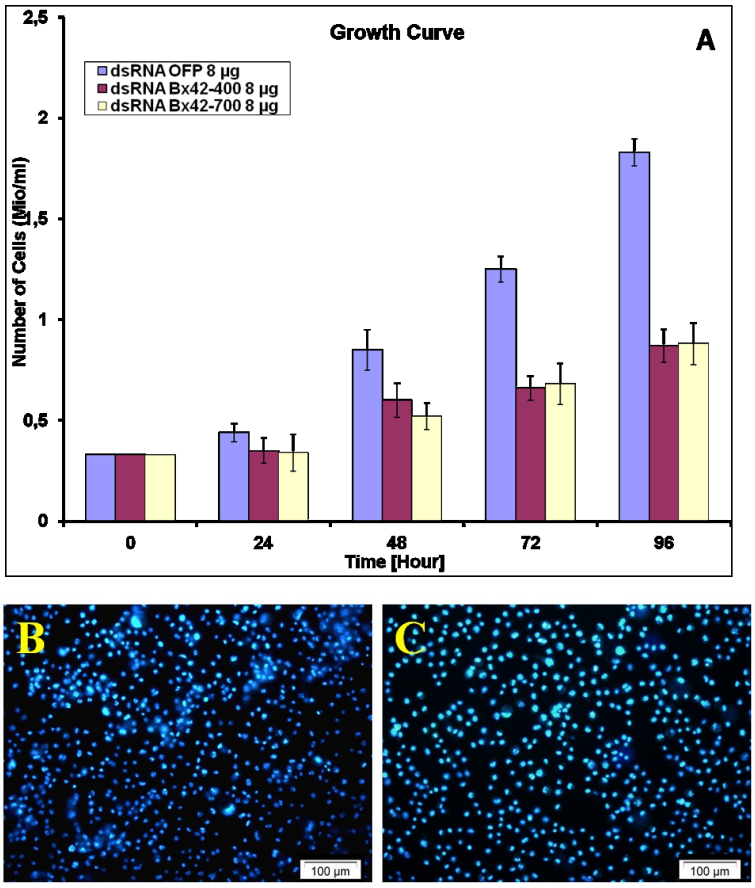


Figure 3-19: The depletion of Bx42 by RNAi inhibits the growth of cells.
(A) S2 cells were plated at a density of $3.3x10^5$/ml, and treated with 8µg dsRNA directed against Bx42-400, Bx42-700 or OFP. Cells were counted at the indicated time points. Error bars represent the standard deviation of three independent experiments. (B) Hoechst staining of control cells (left), and (C) dsRNA Bx42-400 transfected cells (right) at 96h posttransfection. Scale bar 100 µm.

The control cells maintained robust growth. In contrast, cells treated with either dsRNA Bx42-400 or dsRNA Bx42-700 proliferated at rates similar or slightly less to those of dsRNA OFP treated control cells for up to 2 days, but then their growth rate and cell number decreased compared with controls. After 96h of transfection, the cell number of control cells was two times that of dsRNA Bx42-400 or dsRNA Bx42-700 transfected cells. This is shown

in Figure 3-19 A. A representative area of plated control and dsBx42-400 treated cells is shown in Figure 3-19 B, C. As the same results were obtained for both dsRNA Bx42 constructs, I used only Bx42-400 in the follow up experiments, henceforth called dsRNA Bx42.

These experiments were repeated at different concentrations of dsRNA Bx42 (8, 12, and 16µg dsRNA) and the results are shown in (Figure 3-20). Inhibition of cell growth by

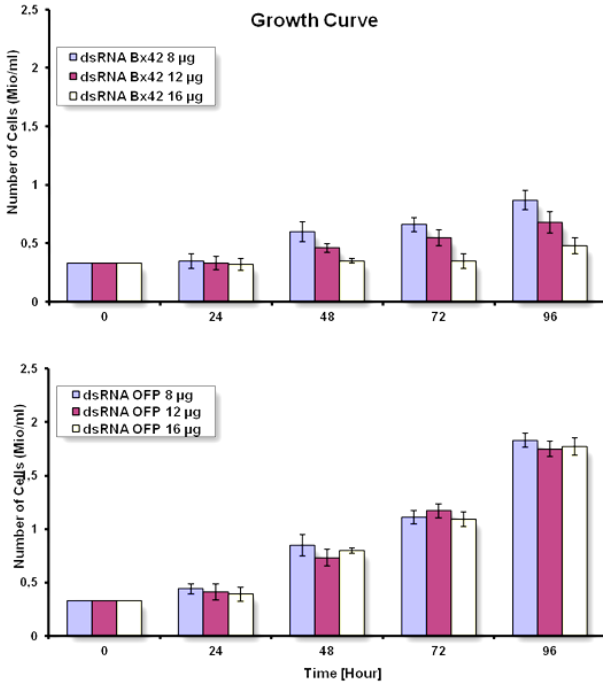


Figure 3-20: The dsRNA Bx42 inhibits the growth of cells in a dose-dependent manner.
Growth curve of S2 cells upon RNAi treatment. S2 cells were plated at a density of 1mio cells/3ml and treated with 8, 12, or 16µg dsRNA directed against Bx42 or an unrelated gene (OFP). Cells were counted at the indicated time points. Error bars represent the standard deviation of at least three independent experiments.

dsRNA was dependent on Bx42 dose as shown in diagram. Using 16µg Bx42 dsRNA resulted almost in arrest of proliferation, whereas the control cells were not affected by any of the used dsRNA concentrations. Adding 8µg and 12µg Bx42 dsRNA resulted in incomplete blocking of all cell growth. Cells treated with 16µg Bx42 dsRNA slightly increased in number also at 96h. This is probably caused by a low percentage of cells that were not transfected. To sum up with, for all following experiments I used 16µg Bx42 dsRNA for transfection.

3.2.1.2 RNAi using Bx42 dsRNA results in efficient knockdown of Bx42 RNA

After transfection with 16µg dsRNA against Bx42 or control OFP, total RNA was extracted from cultures and digested with DNase before analysis. Semi qRT-PCR was conducted 24,

48, 72 and 96h post transfection (Figure 3-21 A). As shown, amount of Bx42 RNA was diminished at 24h post transfection. In addition, gel band intensities indicated that Bx42 transcript was strongly downregulated at 48h post transfection (Figure 3-21).

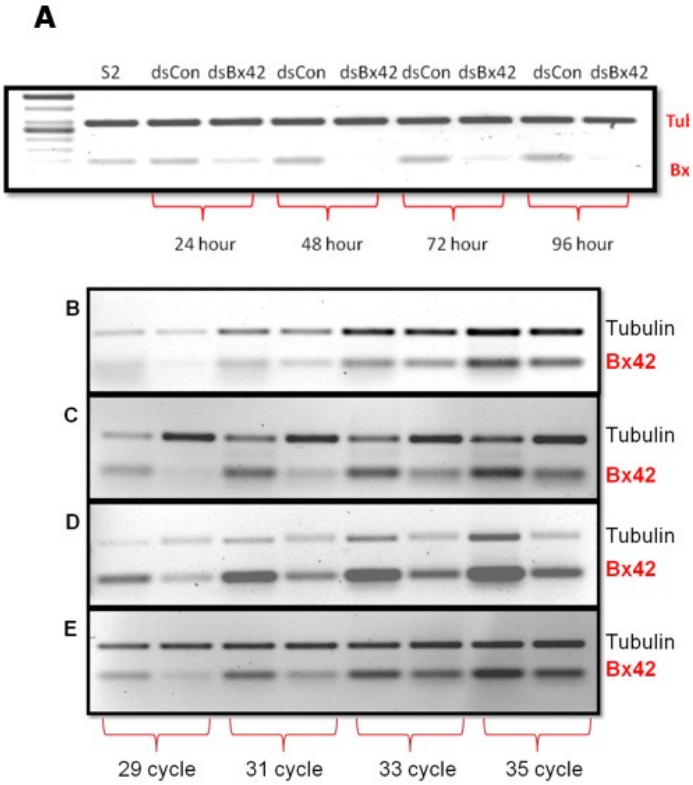


Figure 3-21: Semi qRT-PCR to demonstrate the depletion of Bx42 from S2 cells by dsRNA interference.
(A) The levels of Bx42 cDNA were determined 24, 48, 72, and 96 hours after the addition of dsRNA in control cells and cells treated with dsRNA against Bx42. In the PCR-reaction primer pair's specific for the simultaneous detection of Tubulin and Bx42 were used. (B) Same for RNA 24h following transfection under different PCR-conditions (29-35 cycles as indicated. (C) Same as (B) but RNA 48h following transfection. (D) RNA 72h posttransfection. (E) RNA 96h posttransfection. Tubulin was used as internal standard for the PCR-reaction. The data shown a representative for 3 independent experiments.

3.2.1.3 RNAi using Bx42 dsRNA results in knockdown of Bx42 protein

To detect the changes in Bx42 protein after RNAi transfection, Bx42 rabbit polyclonal antibody was chosen for analysis (Figure 3-22 A). Significant depletion of Bx42 was obtained by RNAi treatment as shown by western blot analysis in which the protein is barely detectable at 96h (Figure 3-22 B).

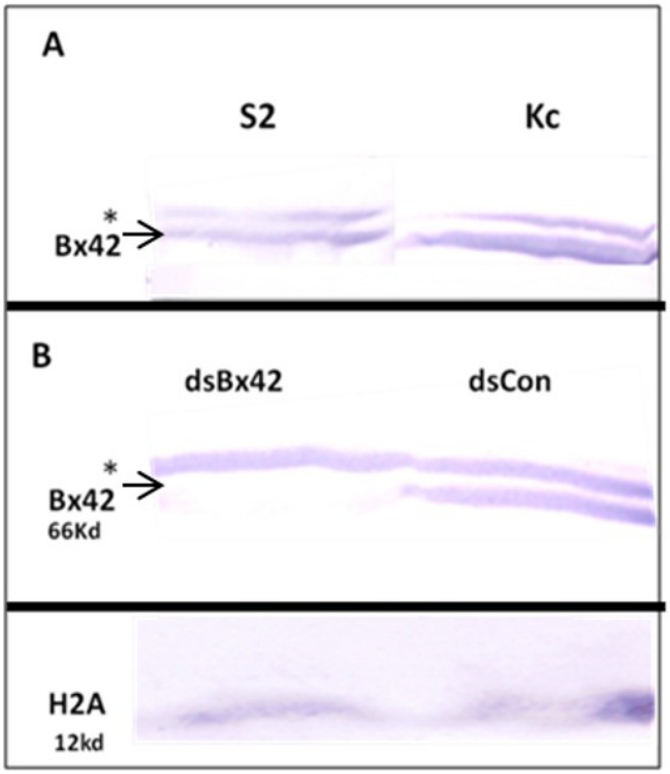


Figure 3-22: Bx42-targeting dsRNA reduced Bx42 Expression.
(A) Western blot of anti Bx42 Rabbit polyclonal on nuclear extracts of S2- and Kc cells. Note that the lower band labelled by an arrow corresponds to Bx42. The band labelled * is an unspecific reacting protein. **(B)** Bx42 was down regulated for 96 h using dsRNA and Bx42 protein was analyzed by Bx42 antisera used in (A). 96h after Bx42-RNAi treatment Bx42 protein was below detection (lane dsBx42), in to the contrast knockdown using dsOFP (lane dsCon). H2A was used as a loading control.

3.2.2 Bx42 depleted cells do not pass through G2/M phase of cell cycle

The arrest of cells could be caused for several reasons, like cell death, mitotic arrest or senescence. Therefore to test these possibilities, I started first to assay the cells for viability in control and Bx42 knock down cells.

3.2.2.1 After Bx42 knockdown most of the cells are still viable.

To test whether the low proliferation rate of dsBx42 transfected S2 cells is a result of cell death, I analyzed the proportion of dead cells using trypan blue staining. Trypan blue is a vital stain used to selectively stain dead tissues or dying cells that take up the dye since their permeability barrier is already affected. My results clearly indicate that Bx42 dsRNAi do not appreciably undergo cell death. Even after 96 h of Bx42 knockdown the percentage of the viable cells was more than 95% compared to untreated control cells that showed $\geq$99% viability or dsOFP-RNA treated cells with 99% viability at this time point (Figure 3-23 A, B).

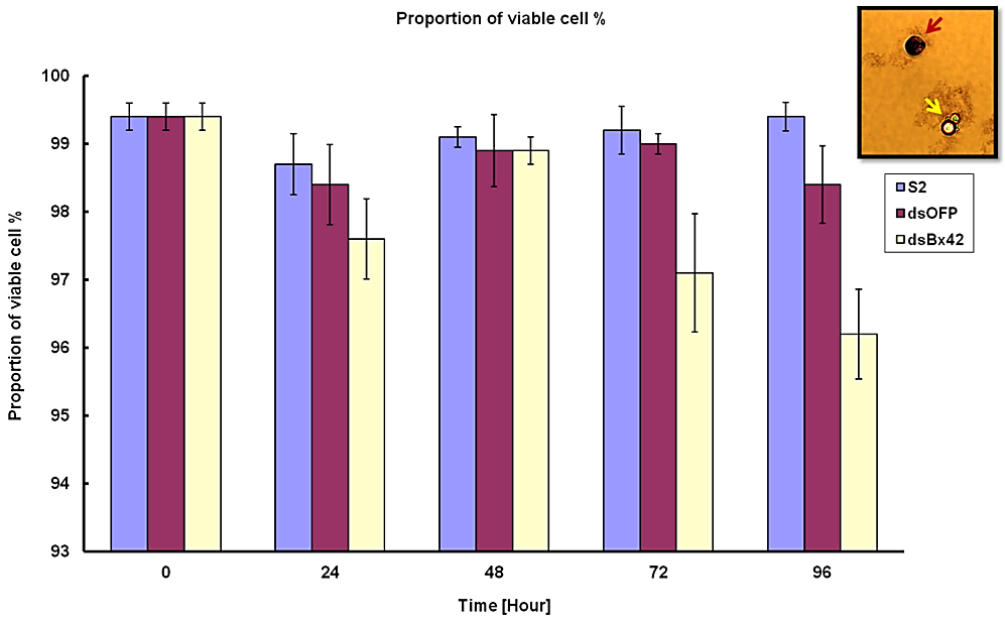


Figure 3-23: Cell viability.
(A)The percentage of viable S2 cells subjected to RNAi. Viabilities were determined by the Trypan blue staining and counted at 24, 48, 72 and 96 hour posttransfection (n=10^3). (B) A figure showed died cell stained with Trypan blue indicated with red arrow and a viable non-stained cell indicated with yellow arrow.

3.2.2.1 EdU staining decreases at 96h following Bx42 RNAi knock down.

Next, I tested whether the Bx42 knockdown cells arrested in the cell cycle. If so, it was important to determine the exact stage of cell cycle where these cells arrested. To do this, I first used a 3h pulse labeling by EdU as a measure of the proportion of cycling cells undergoing S phase. EdU was added to cells 96h after dsRNA treatment (16 µg). 3 hours later cells were fixed and permeabilized so that EdU incorporated during this time could be visualized. Less than 5% of the dsBx42 transfected cells incorporated EdU, while more than 40% of control cells were labeled (Figure 3-23). When I repeated the EdU incorporation experiments with cells 24h and 48h posttransfection I found no decrease in EdU incorporation compared to the controls (dsBx42-RNA: 50% EdU labeled cells at 24h, and 56,7% EdU labeled cell at 48h knockdown; dsOFP-RNA: 38% EdU labeled cells at 24h, and 40% EdU labeled cells at 48h knockdown; Figure 3-24). In fact, at earlier time points the proportion of EdU labeled cells on dsBx42-RNA knockdown even seemed to slightly increase in comparison to dsOFP-RNA control cells. Representative areas of cells stained by EdU at different times posttransfection are shown in (Figure 3-25 A-F′) for the control and Bx42 RNA. Whereas clear EdU signals are present in a significant proportion of Bx42-dsRNAi treated cells at 24 and 48h posttransfection, the proportion of EdU stained cells is dramatically decreased at 96h. In contrast, OFP-dsRNA treated cells show a constant ratio of EdU staining cells whose number stably increases with time.

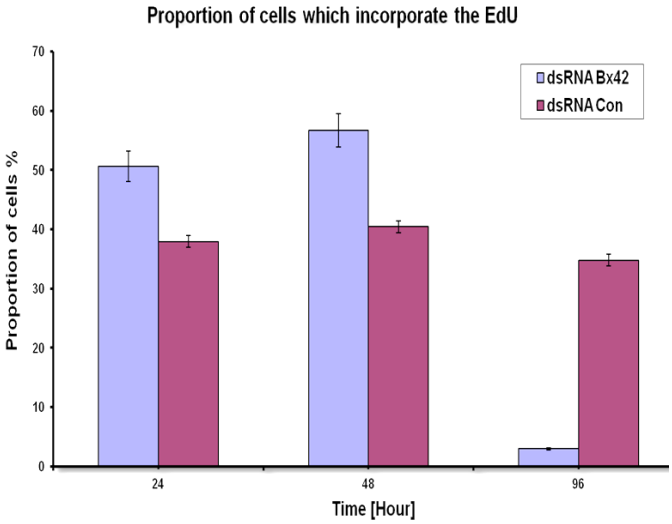
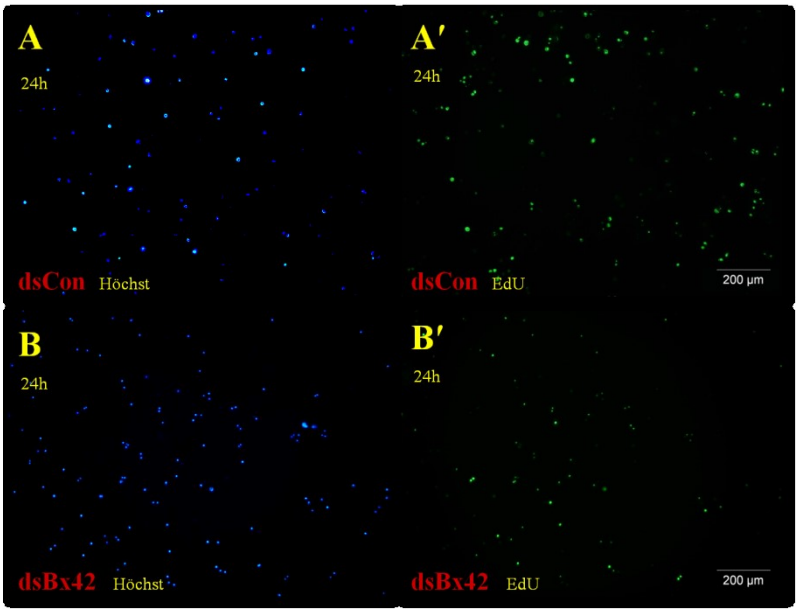


Figure 3-24: Bx42 depletion by RNAi shifts the proportion of S-phase cells relative to the control.
Cells following dsBx42-RNA or dsOFP-RNA (control) knockdown were treated by EdU for 3h following dsRNA for the time indicated, then counted. Error bars represent the standard deviation of three independent experiments.



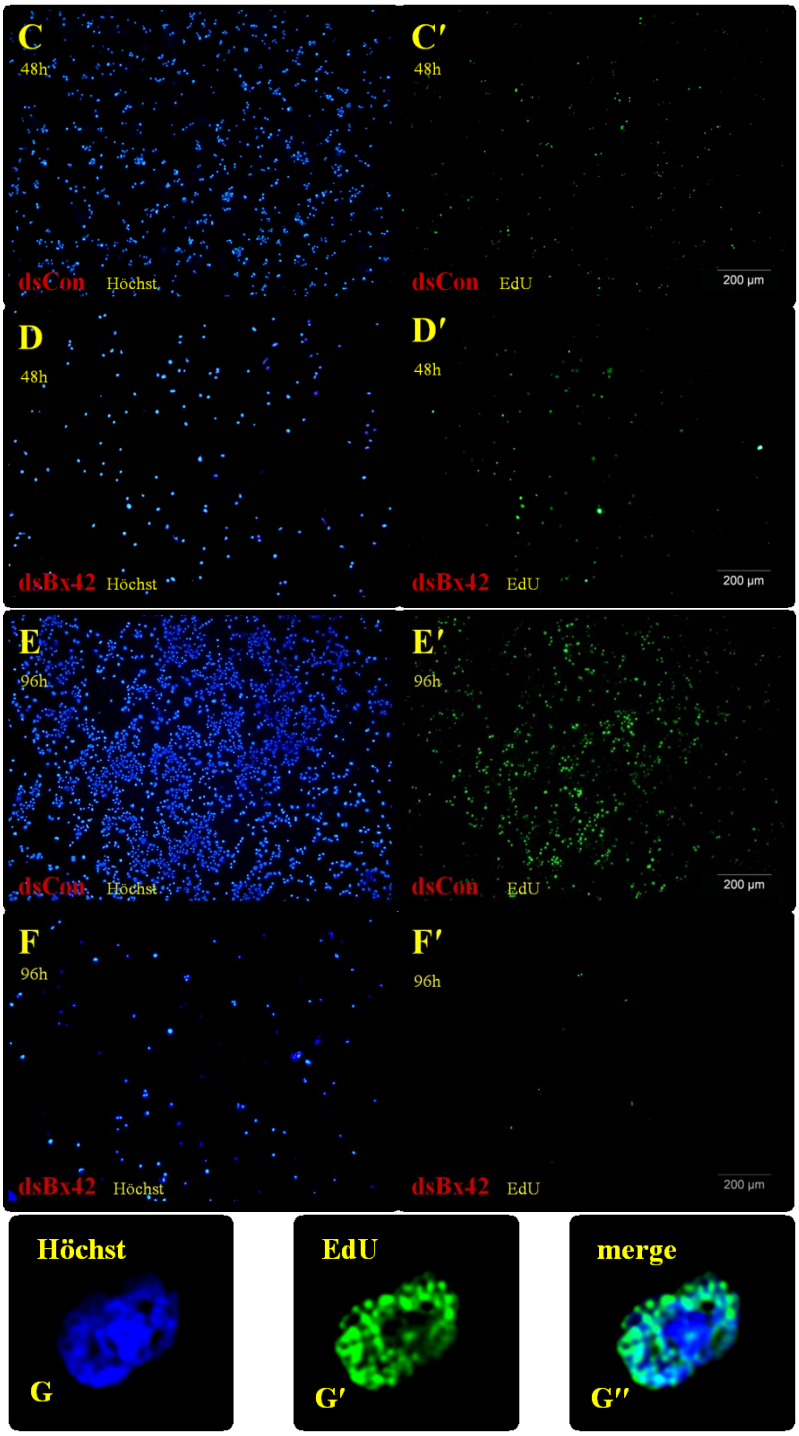


Figure 3-25: Bx42 depletion by RNAi shifts the proportion of S-phase cells relative to the control.
EdU incorporation and Hoechst staining of control and dsBx42- treated cells at 24h (A, A′ dsCon and B, B′ dsBx42) , 48h (C, C′ dsCon and D, D′ dsBx42) ,and at 96h (E, E′ dsCon and F, F′ dsBx42); note also the increase in cell number over time of dsOFP-RNA treated controls compared to dsBx42 RNA treated cells. Bar: 200µm. G-G`` shows a higher magnification of Hoechst and EdU stained cell nucleus photographed by Delta Vision Microscope (G, G′, G″).

3.2.2.2 Phosphohistone staining decreases following Bx42 RNAi knock down.

Cell cycle could also be arrested at G2/M transition. To test this, I carried out immunostaining and Western blotting of dsRNA treated cells using an antibody specific to phosphohistone H3S10. The immunostaining of dsRNA Bx42 depleted cells indicates that

in contrast to S-phase cells the number of phosphohistone H3S10p stained cells begins to decrease earlier, already at 24h post transfection. I observed a reduction of H3S10p stained cells by ~33% compared to the OFP control and almost no such cells were observed at 96h post transfection (Figure 3-26 A). OFP-RNAi controls over the same time retain a constant rate of mitotic cells (Figure 3-26 A; Figure 3-27 A-D′). Western blotting of asynchronous cultures of control or RNAi-treated cells indicated a similar decrease in the level of phosphorylated histone H3S10p in dsRNABx42 transfected S2 cells compared to the OFP control at 96h post transfection (Figure 3-26, B).

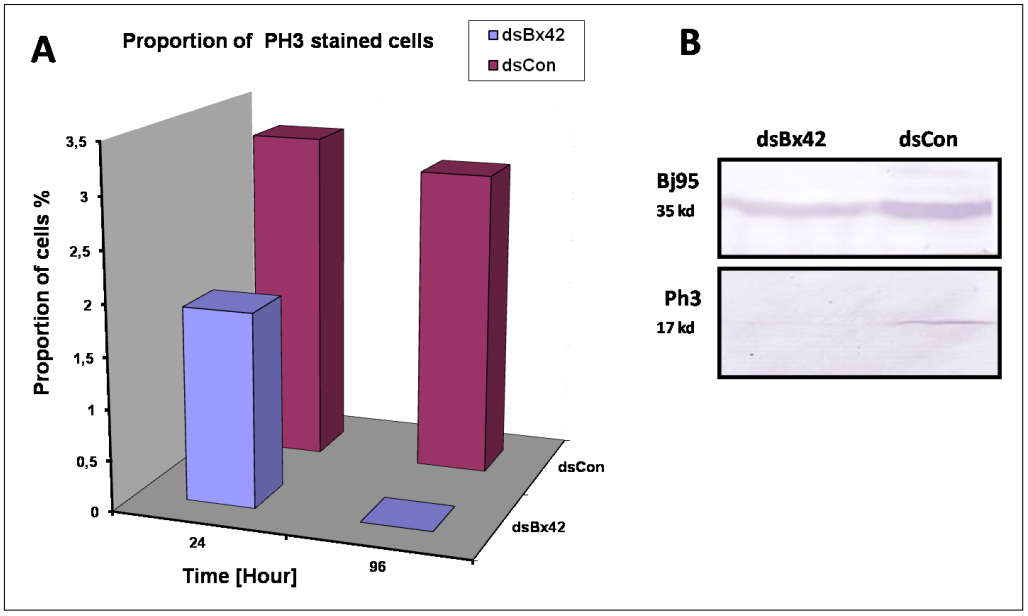


Figure 3-26: The Bx42dsRNA cause reduction in the rate of M-phase cells.
(A)Diagram shows the percentage of H3S10-P in control and Bx42dsRNA transfected S2 cells at 24 and 96h post transfection. (B) A Western blot that shows reduction in H3S10-p staining (bottom) in dsRNA Bx42-treated cells compared with control; H1 stained by the monoclonal antibody Bj95 was used as a loading control.

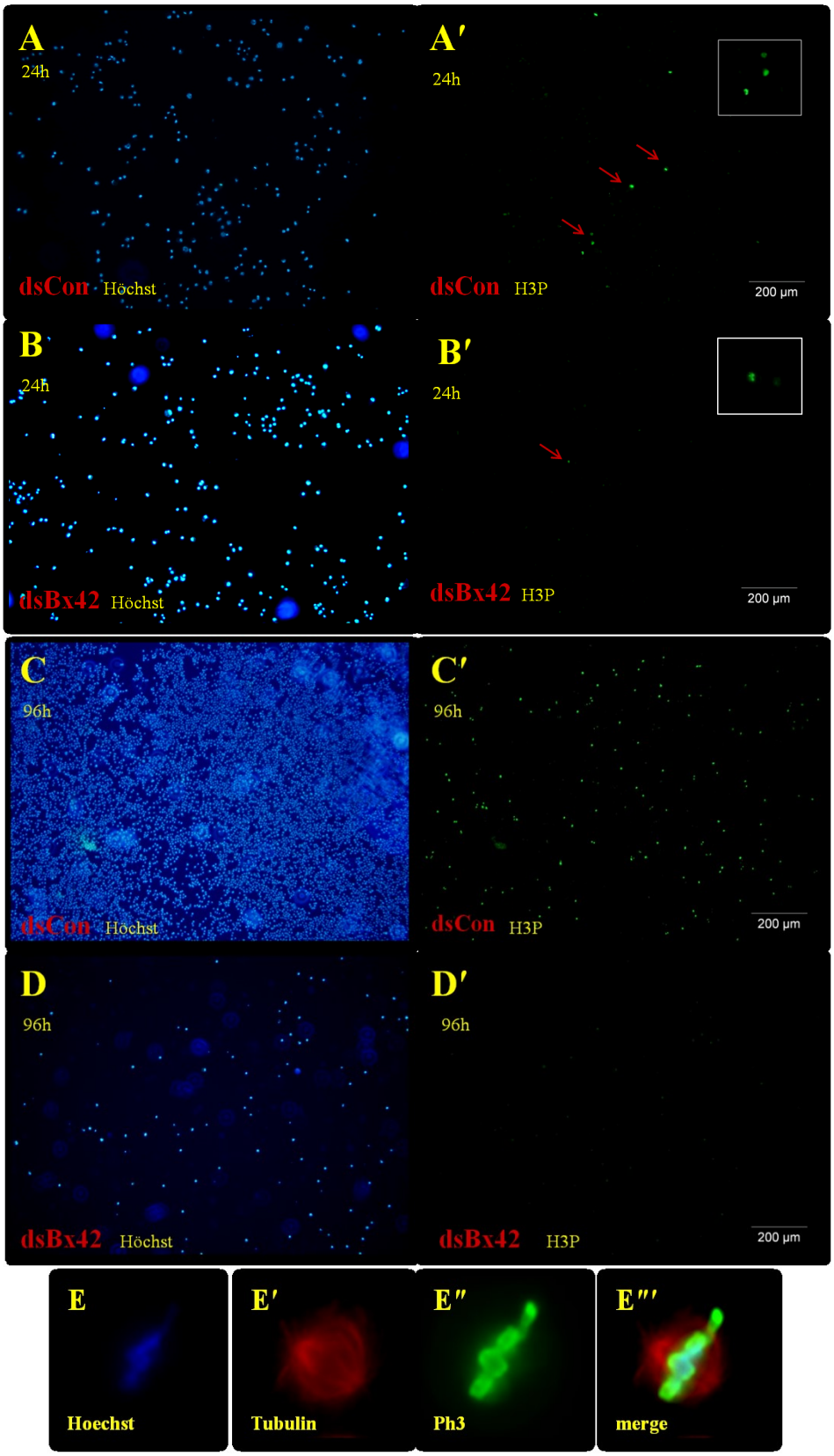


Figure 3-27 : Histone H3 phosphorylation in Control and Bx42 RNAi S2 cells.
The histone H3 phosphorylation revealed by anti phospho-histone H3 antibody (green) and DNA (blue) staining ; A, A′ dsOFP control, B, B′ dsBx42 at 24h; C, C′ dsOFP control, D, D′ dsBx42 at 96h. Bar 200µm. (E, E′, E″, E‴) Single mitotic cell at high magnification stained with Hoechst (blue), Tubulin (red), anti phospho-histone H3 antibody (green); and merge respectively.

3.2.2.3 FACS results from DNA stained S2 cells indicate an increased cellular DNA content following Bx42 RNAi.

The results I got so far show a dependency of the cell cycle progression on Bx42. I wished to obtain more detailed information by studying the cell cycle phases of dsBx42 RNAi transfected cells. Therefore, I analysed the DNA profiles using flow cytometry. Cells were treated with Bx42 dsRNA and with OFP- control dsRNA, stained with propidium iodide at 24, 48, 72, and 96h post transfection and analysed using a flow cytometer.

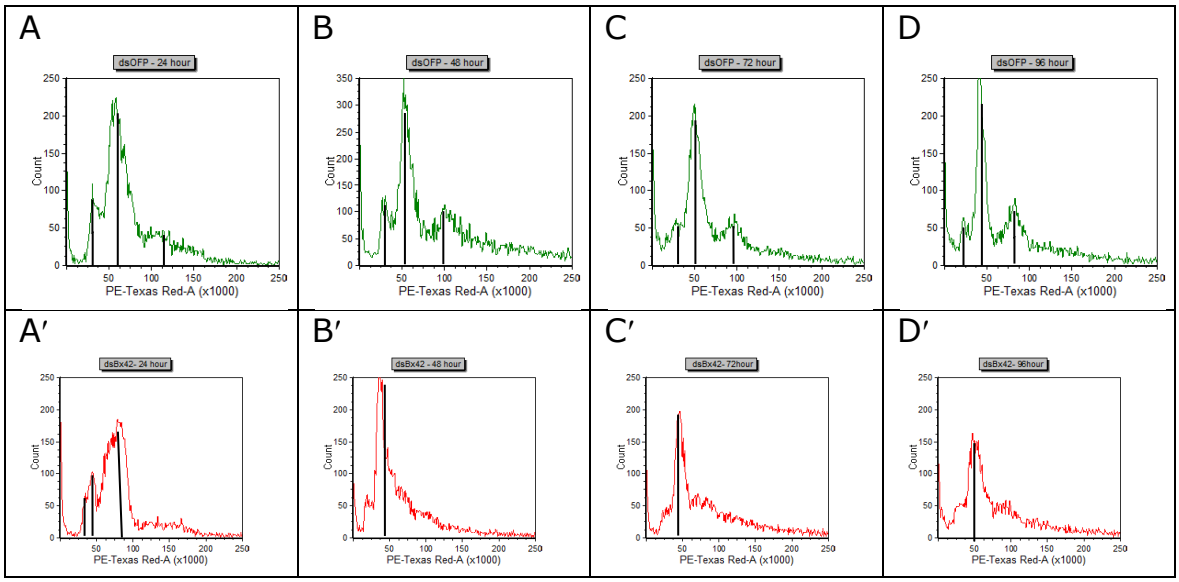


Figure 3-28: Depletion of Bx42 impairs cell proliferation and leads to S-phase se cell cycle arrest.
(A, B, C, D) FACS analysis of asynchronously growing S2 cells at 24, 48, 72 and 96h. Cells were treated with the indicated dsRNA, stained with propidium iodide and analyzed using a flow cytometer. Upper three diagrams in green dsOFP-RNA controls, lower three diagram dsBx42-RNA treatment; times of dsRNA exposure indicated at the top of each diagram.

The line charts shown in (Figure 3-28 and Figure 3-29) demonstrate an example of the results obtained. The analysis of cell cycling with S2 cells is somewhat obscured by the fact that these cells are aneuploid to a certain proportion. This may explain the three peaks found in the OFP. The first peak at ~30 may correspond to euploid G1 cell fraction, the second, large peak at ~ 50 reflects the aneuploid G1 cells and third peak at 100 reflects the position of G2 cells. Such a profile can be observed for the OFP control at any time point following transfection. In contrast, the profile of Bx42dsRNA treated cells already differs 24 after transfection. The first peak is shifted relative to OFP control and has a maximum at the position ~45 suggesting that this broad peak reflects euploid cells in S-phase.

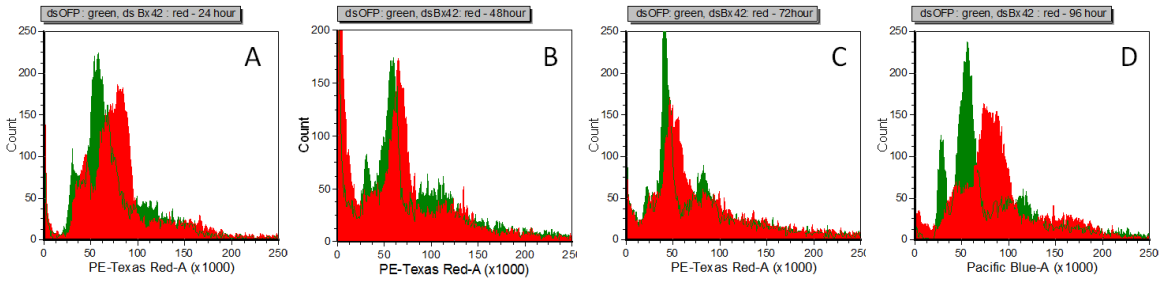


Figure 3-29: Depletion of Bx42 impairs cell proliferation and leads to S-phase se cell cycle arrest.
(A, B, C, D) FACS analysis of asynchronously growing S2 cells at 24, 48, 72, and 96h. Cells were treated with the indicated dsRNA, stained with propidium iodide and analyzed using a flow cytometer; overlay of graphs shown in figure 3-27; green dsOFP control, red dsBx42

The second broad peak with a maximum that is shifted relative to the OFP could indicate the position of aneuploid cells initiating S-phase and may include aneuploid G1 cells. There is no accumulation of G2 cells at that time. Later 48-96 posttransfection, the first peak disappears, suggesting that these cells may be arrested in S or G2 phase. The second peak first 48h increased and then decreased 72 and becomes broader and shifted to a range between late S-phase or G2 cells at 96h suggesting that with time most cells leave G1 and arrested in late S-phase or G2 although a clear G2 peak as seen in the control never forms for Bx42ds treated cells.

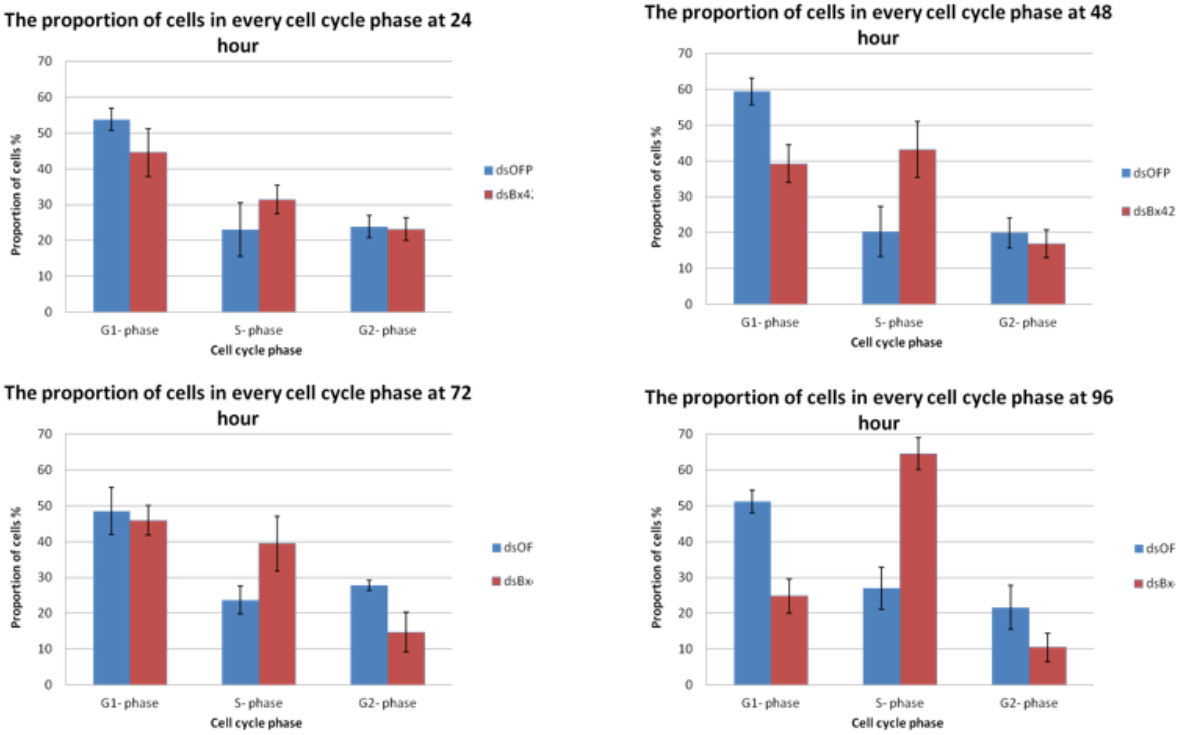


Figure 3-30 Depletion of Bx42 impairs cell proliferation and leads to S-phase se cell cycle arrest.
The proportion of cells in G1-, S- and G2- phase at 24, 48, 72, and 96 hours post transfection; data are the means of three independent FACS experiments.

I also used the FACS profiles for quantitative evaluation of the behavior of the dsRNA treated cells. The ratio of cells in different stages of cell cycle for different time points posttransfection is shown in the bar charts in (Figure 3-30), these data were quantified using De Novo- FCS 4 Express flow plus image software. In the control the ratio of G1 phase cells is ~2-fold the ratio of S-phase or G2 respectively (G1 48-60%; S 20-26%; G2 20-28%). In the Bx42 knockdown cells the ratio of G1 phase cells is decreasing, G1 phase cells is decreasing from 45% at 24h post transfection to 24% at 96h posttransfection. In contrast, the ration of S-phase cells increase from 30% at 24h posttransfection to 64% at 69h posttransfection. This again indicates problem for the Bx42 dsRNA cells to leave S-phase.

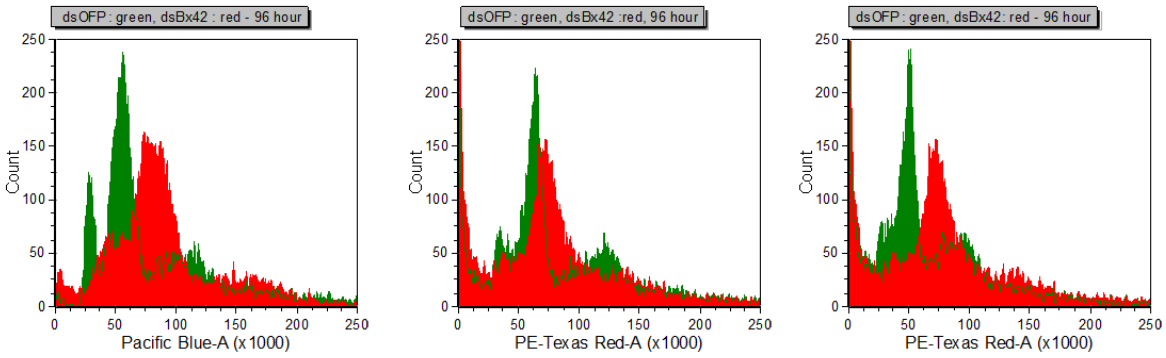


Figure 3-31: Overlay of three independent FACS experiments comparing dsOFP and dsBx42 ds(RNA) cells at 96hour post transfection. The overlay of three independent FACS experiments 96h posttransfection again show the relative increase of S-phase cells of Bx42-RNAi (red) compared to controls (green).

The relative decrease in G1 and G2 cells and increase in S-phase cells of Bx42-RNAi in comparison to the OFP control is illustrated in (Figure 3-31), which represents three diagrams of 3 independent FACS experiments of control and dsBx42 depleted cells at 96h posttransfection. It shows that the Bx42 RNAi transfected cell cycle shifts dramatically from distinct peaks in G1 and G2 phases of the control cells to cells mostly in S-phase, as depicted by the location of the peak in between G1 andG2. It can be concluded that, the dsBx42 transfected cells seemed to have a problem to pass through S-phase.

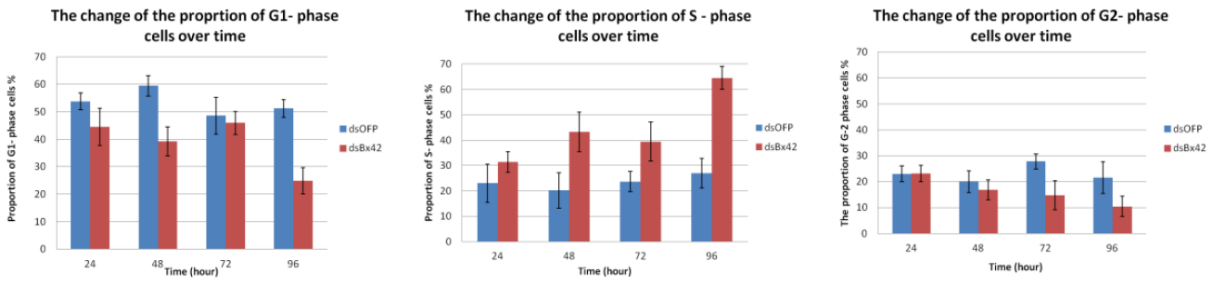


Figure 3-32: The changes in the proportion of G1, S, and G2 phase cells over 96 hours post-transfection.
These data are the mean of the three independent experiments shown in figure 3-30. On the X-axis, information on dsBx42 and control transfected cells is given with respect to time (hour post transfection). The Y-axis quantifies the percentage of cells in G1-, S-, or G2-phase.

To better compare the changes for given cell cycle phases over time after knockdown, the same results were allocated to presumed phases of the cell cycle and presented in a bar chart as seen in (Figure 3-32). For the G1 phase Figure 3-32 shows that, in general the percentage of G1-phase cells in the OFP control remains constant since it ranges between 48% and 60%. On the other hand, it can be noticed that the percentage of G1- cells following dsBx42 treatment decreased dramatically from 45% at 24h to 24% at 96h posttransfection. In contrast, the middle bar chart (Figure 3-32) demonstrates that the proportion of S-phase cells following dsBx42 transfection steadily increased from 30% at 24h to 64% at 96h. The control cells on the other hand remained at 20-26% throughout time. The G2 cells also steadily decreased from 23% at 24h to 10% at 96h and reached a low point of 10% less than the half of control Bar chart (Figure 3-32).

By observing the graphs given in Figure 3-32, it can be concluded that, there is general decrease in G1 and G2 cells and an increase in S-phase cells with increasing time of knock down. I also want to emphasize, that this tendency is somewhat corrupted at 72h for G1-phase cells and at 48h for the S-phase cells. An explanation for these “outliers” may be the somewhat uncertain allocation of cells to a specific phase in the cell cycle.

3.2.2.4 Following Bx42 RNAi knockdown there is a slight decrease in cell viability.

One reason for the diminished cell number following Bx42 knockdown could be the induction of cell death. As already mentioned, trypan blue staining did not give evidence for significant increase cell lethality under these condition. To proof that depletion of Bx42 does not cause apoptosis, *caspase-3* activity was measured at the transcription level and at the protein level using antibodies against cleaved caspase-3. S2 cells were treated with dsRNA targeting Bx42 and harvested 24, 48, 72 and 96h later. Semi q RT-PCR showed no differences for *caspase-3* mRNA between OFP controls and Bx42 depleted cells (Figure 3-33 A).

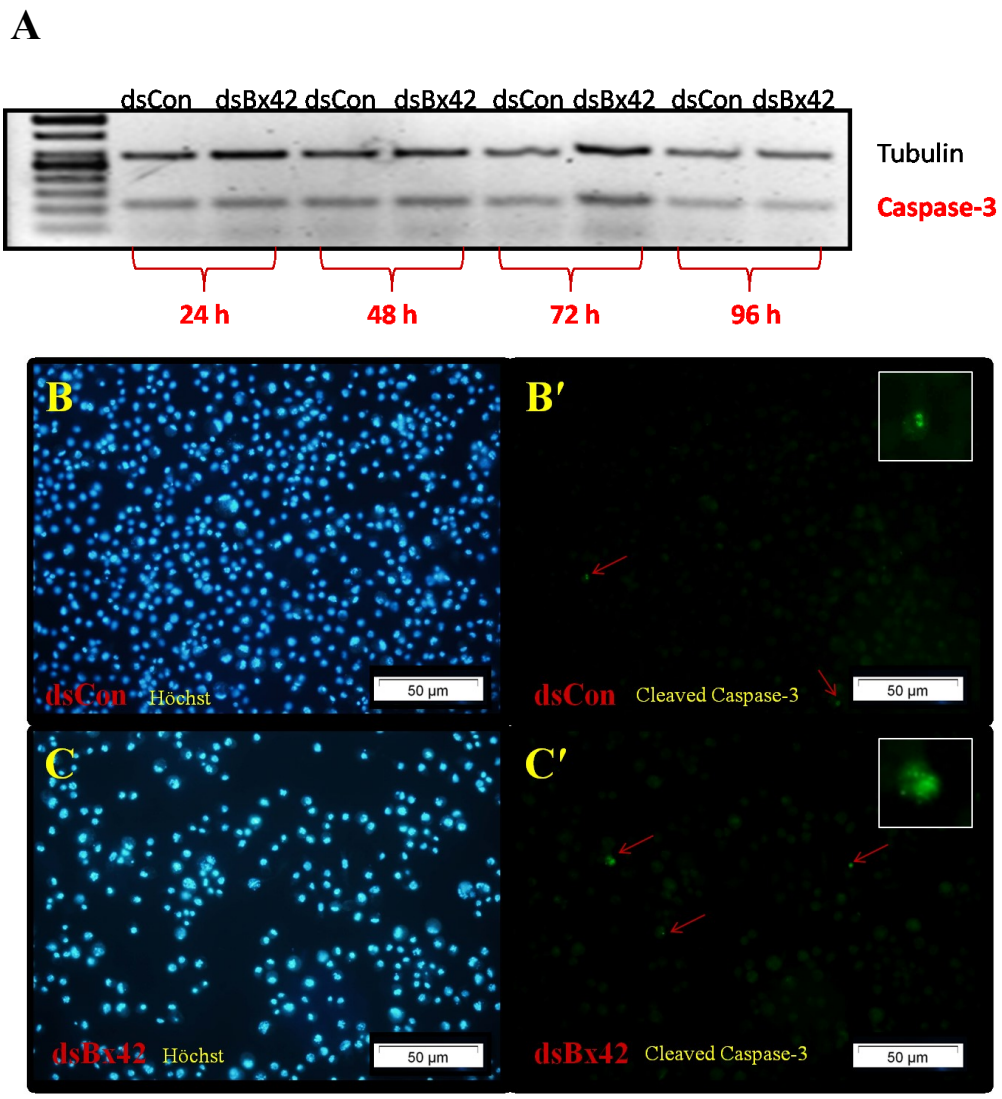


Figure 3-33: dsBx42 transfection of S2 cells does not result in cell death.
(A) RT-PCR, total RNA was isolated from cells transfected with dsRNABx42, and dsOFP. To amplify the endogenous Tubulin and Caspase-3 transcripts, a multiplex RT-PCR was performed using primers from Tubulin and Caspase-3. The samples were gathered at 24, 48, 72, and 96h post transfection. All experiments were performed in triplicate, and a representative sample is shown. (B-C′) dsRNA transfected S2 cells grown at 25°C for 4 days. Cell death for transfected S2 cells was quantified by cleaved caspase-3 immune staining. dsRNA Bx42 transfected S2 cells (C, C′) compared with controls (B, B′). Bar 50µm.

Immune staining using an antibody against cleaved activated Caspase-3 showed a slight increase in apoptosis (Figure 3-33 B- C′). The proportion of cells undergo apoptosis was 7,7% in dsRNA-Bx42 S2 cells comparing with 0,7% of the control (n=10^3 cells were counted in both cases).

3.2.2.5 ß-galactosidase staining indicates that the arrested cells do not undergo senescence.

Another reason causing lack of proliferation is senescence, a viable non-proliferating state characterized by an inability to return into the cell cycle. Several markers of senescence were described, among them are cytological markers such as senescence-associated heterochromatin foci (SAHF), as well as cell structural or enzymatic changes such as increase in cell size and increased lysosomal ß-galactosidase activity detected at pH 6,0 and defined as senescence-associated ß-galactosidase (SABG), both often used as markers to identify senescent cells. However, Bx42-S2 transfected cells did not show any characteristic change in nuclear morphology and did not develop the senescence-associated heterochromatin foci (SAHF- data not shown). Also, senescence-associated ß-galactosidase (SABG) staining showed no significant increase in the proportion of senescent cells in Bx4-dsRNA transfected cells (0,86%) in comparison with control cells (0,33%) (Figure 3-34).

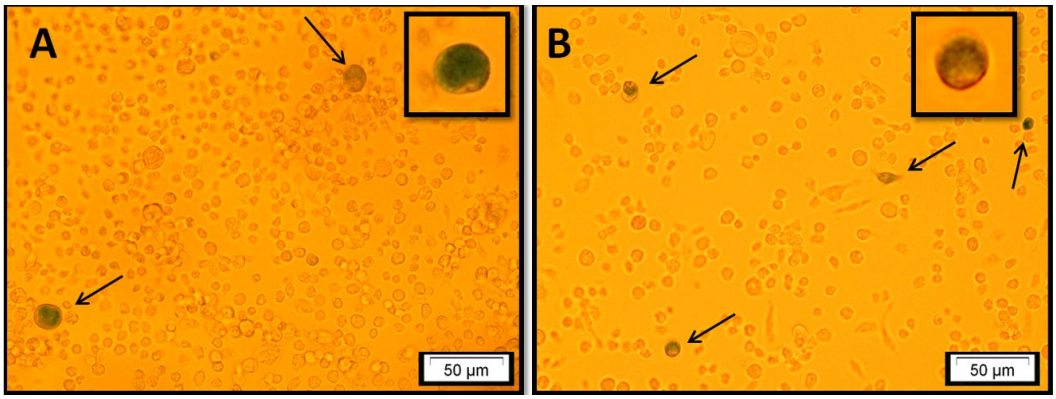


Figure 3-34: dsBx42 transfection of S2 cells does not result in expression of SABG senescence marker.
Transfected S2 cells grown at 25°C were incubated 4 days before senescence was quantified by ß-galactosidase staining (A) dsOFP-and (B) dsBx42-transfected cells. Bar 50µm.

Another indicator for senescence is the tumor suppressor p53 protein. This protein is well known for its role in DNA repair and apoptosis, and has a multitude of links with the cell cycle (Blomen & Boonstra, 2007). I performed semi qRT-PCR for P53 expression but failed to detect any differences in the levels of p53 mRNA between Bx42 transfected cells and the OFP control cells (Figure 3-35 A). However, the immune staining using antibody against P53 showed an 20x increase in P53 positive cells in Bx42 transfected cells (6,4%) compared with

control cells (0,32%) (Figure 3-35 B, C). Probable reasons for the differences between qRT-PCR and immunostaining results will be discussed later.

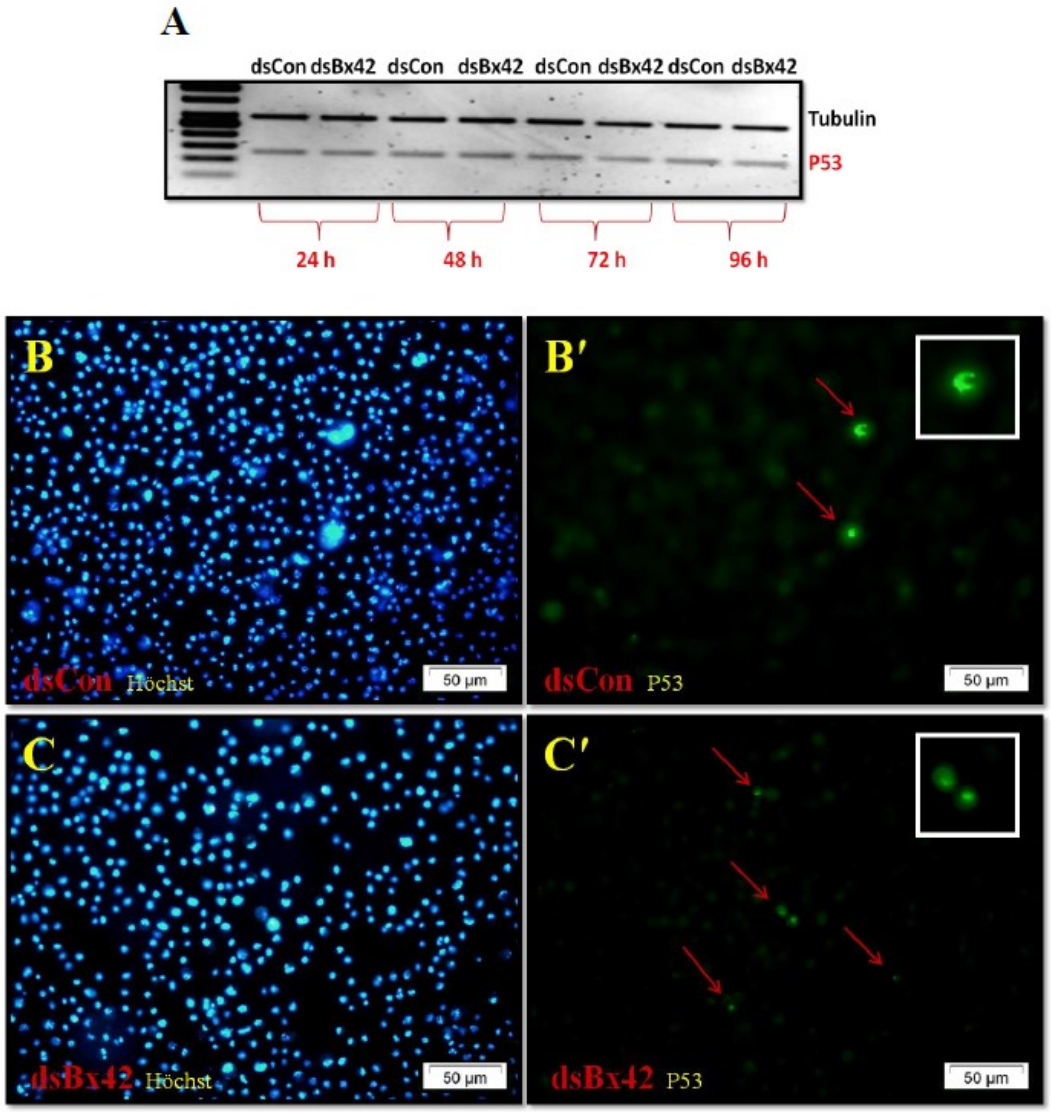


Figure 3-35: P53 is expressed in dsBx42 transfected S2 cells.
(A) Semi qRT-PCR: Total RNA was isolated from cells transfected with dsRNA Bx42 and dsRNA OFP and collected at 24, 48, 72 and 96h post transfection. To amplify the endogenous Tubulin and P53 transcripts, a multiplex RT-PCR was performed using primers from Tubulin and P53. All experiments were performed in triplicate, and a representative gel is shown. B-C′: Transfected S2 cells were grown at 25°C for 4 days. P53 immune staining of OFP-control (B, B′) and dsBx42 transfected cells (C, C′). Bar 50µm.

3.2.3 Bx42 RNAi results in down regulation of cell cycle regulators.

3.2.3.1 Semi qRT-PCR shows down regulation of Cyclin A, Cyclin B, E2F and Dacapo RNA

Next, using semi qRT-PCR method I examined the expression of the main cell cycle-related genes in dsRNA treated S2-cells (Figure 3-35). Bx42 depletion resulted in reduction of E2F transcripts from 24h post transfection, at 48h it almost undetectable and a significant reduction was also observed at 72- 96h post transfection (Figure 3-36 A). In a similar fashion, the mRNA levels of Cyclin A and Dacapo began to decrease in Bx42 depleted cells from 24h post transfection as compared to controls, but decreased significantly at 48h. Small amounts of Dacapo transcripts appeared at 72 h, and more obviously at 96h post transcription, cyclin A transcript was still undetectable at 72h and was detected at 96h post transfection (Figure 3-36 B, C).

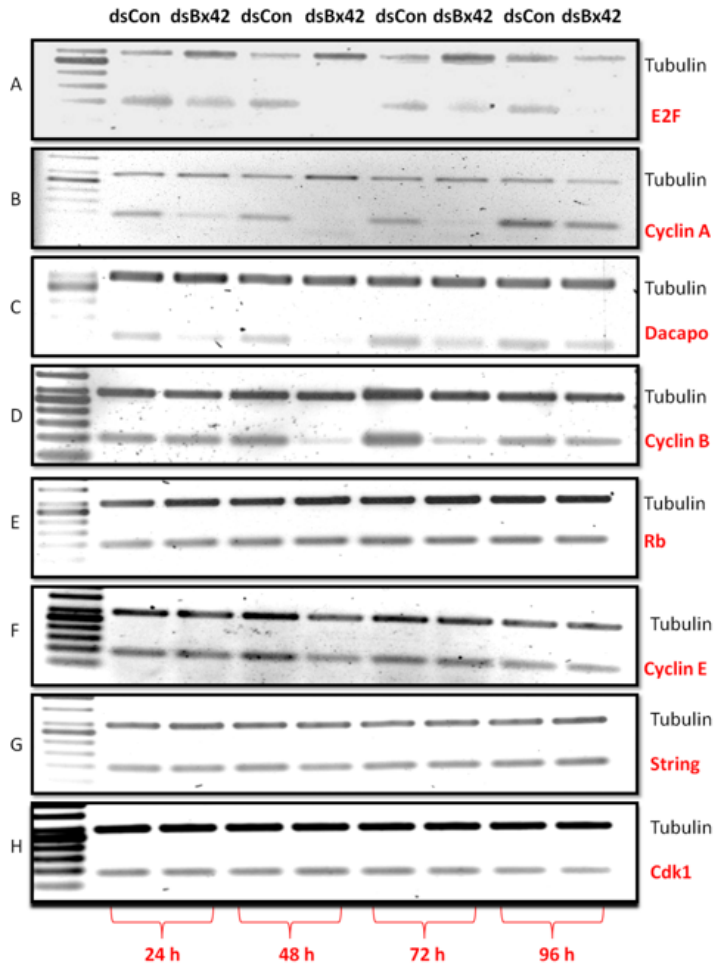


Figure 3-36: The effects of dsRNA Bx42 on cell cycle-related genes in S2 cells.
Total RNA was isolated from cells transfected with dsRNA Bx42 and dsRNA OFP and collected at 24, 48, 72 and 96h post transfection RTPCR of target genes was carried after DNase digestion. The gene-specific primers used for PCR are listed at the far right of each figure.

Transfection with dsBx42 did not result in significant changes in the Cyclin B mRNA levels at 24 h as compared to controls but decreased significantly at 48 and slowly increased at 72 and 96h post transcription (Figure 3-36 D).

These early effects of Bx42 depletion detected at 24/48h post transcription suggest that Bx42 protein half-life is shorter as 24h that its transcripts began to disappear at 24h post transcription and the consequences of this reduction could be detected as early as 24h post transfection. Secondly, Bx42 seemed to be directionally implicated in the transcription (or splicing) of the cell cycle regulators E2F, Cyclin A, Cyclin B and Dacapo. In contrast to that, no change was observed in the levels of Rb and Cyclin E transcripts in dsBx42 transfected cells in comparison to dsOFP control cells (Figure 3-36 E, F). Additionally, transcript level of String, the *Drosophila* homolog of *cdc25* mitotic activator, was detected and no change was shown (Figure 3-36 G). Cdk1 was identified as Bx42 interaction partners in the eye genetic screen in the first part of this work. Therefore, Cdk1 transcript level was also detected, and no change was observed overtime (Figure 3-36 H).

3.2.3.2 Western blot in S2 cells shows down regulation of Cyclins A and B following Bx42 RNAi

To further test the effect of Bx42 deficiency on Cyclin A and Cyclin B, I examined the protein levels of Cyclin A and Cyclin B as well as Cyclin E proteins in the Bx42 knockout cells 96h post transfection. The results suggest that Bx42 deficiency induces down regulation of Cyclin A and Cyclin B (Figure 3-37) but not Cyclin E.

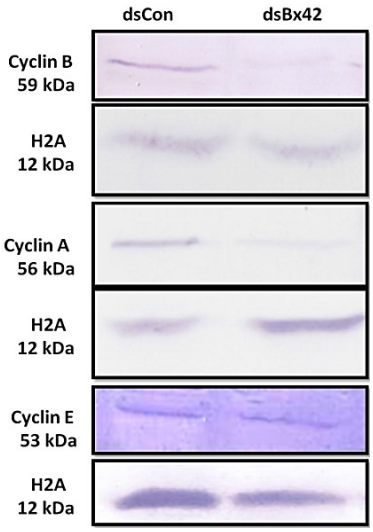


Figure 3-37: *Drosophila* Bx42 silencing cause down regulation of Cyclin A and Cyclin B but not Cyclin E in S2 cells.
Protein analysis of equal amount of cell lysates from dsCon and dsBx42 transfected cells 96h post transfection was analyzed by immunoblotting.

4 Discussion

In the present study, I aimed to determine the role of *Drosophila* Bx42 in the control of cell proliferation in *Drosophila* eye development and *Drosophila* Schneider S2 cells. The results of my experiments with the *Drosophila* eye showed that, expression of the dominant negative Bx42-SNW in the late 3rd instar eye discs results in a reduction of eye size corresponding to a reduction of the number of the mitotic cells at the second mitotic wave and an elevated cell death in the posterior part of eye discs of GMR-GAL4/UAS-Bx42-SNW animals. A genetic screen aimed at genes involved in cell cycle regulation that modify the Bx42-SNW small eye phenotype identified modifiers as signaling pathway and cell cycle regulator genes. Moreover, the expression of the main cell cycle regulators has been studied and a reduction of Cyclin A and Cyclin B could be shown in the eye discs of GMR-GAL4/ Bx42-SNW animals. In the second part of this study, RNAi knockdown method was applied to study the effects of Bx42 depletion in Schneider S2 cells on cell proliferation. Bx42 RNAi transfection into S2 cells results in arrest of cell growth. Further analysis showed that these cells have a problem to pass through S-phase to G2. Following Bx42 RNAi knockdown there was only a small decrease of about 5% in the proportion of viable cells compared to controls. Additionally, ß-galactosidase staining indicates that senescence was only slightly elevated. Furthermore, Bx42 RNAi induction in S2 cells resulted in downregulation of major cell cycle regulatory proteins as was found in the study of eye development, the first part of this work.

4.1 Investigating the role of SKIP/Bx42 in *Drosophila* eye

4.1.1 Bx42 is required for normal eye growth

4.1.1.1 Evidence that Bx42-SNW is dominant negative involved in cell proliferation

The function of *UAS Bx42-SNW* in eye growth control was investigated using the GAL4-UAS system. Four eye-specific driver lines were used and the results of these crosses were different depending on the driver line. One of them was *ato*-GAL4.

The proneural *atonal* (*ato*) gene that is expressed ahead and within the morphogenetic furrow to specify R8 precursor's cells is regulated by several signaling pathways. During the progression of the morphogenetic furrow, Hh signaling activates at least two different signals. One is Dpp, a long range signal that primes cells to adopt a ‘pre-proneural’ state. These cells are ready to initiate neural differentiation, because they express Atonal and Daughterless, but are held in check because they also express the inhibitors Hairy and Extramacrochaetae (Emc),

which keep the *atonal* expression and activity at low levels. Delta/Notch activation provides a second Hh-dependent signal, which only works at short range within the morphogenetic furrow, as Delta is a membrane-bound ligand. The proneural action of Notch signaling increases Atonal activity by two mechanisms: *atonal* is transcriptionally upregulated, and at the same time a repressive co-factor is removed by downregulation of Hairy and Extramacrochaetae (Baonza and Freeman., 2001). These concerted actions lead to the accumulation of active Atonal and thereby the initiation of neural differentiation. A different short range signal, unidentified but induced by Raf, has also been proposed to upregulate Atonal (Greenwood and Struhl, 1999). In fact, the results of (Baonza and Freeman., 2001) point to the existence of at least one proneural signal other than Dpp and Notch: the initial expression of *atonal* can be induced even when both Dpp and Notch signaling are simultaneously blocked. They have suggested that this other signal may be Hh itself, acting directly to activate *atonal* expression, as has been proposed by Domínguez (Domínguez, 1999; reviewed by Voas and Rebay., 2004). The failure of obtaining an eye phenotype following *ato*-GAL4 dependent Bx42-DN activation may be explained by the assumption that Bx42 is not at all needed in the Notch dependent *atonal* expression. However, the cause for this negative result may be due to the low expression from the *ato*-Gal4 driver line itself.

A possible explanation of the lethality caused by expression of *UAS Bx42-SNW* using the second driver line *elav*-Gal4 may be that *elav* plays an early role in neurogenesis. *Bx42* was identified as a gene required for *Drosophila* nervous system development in an RNAi study by Ivanov and his coworkers (2004). Therefore, overexpression of the dominant negative allele *UAS Bx42-SNW* in the nervous system using *elav*-Gal4 may result in defect neurogenesis and embryonic lethality. Actually, a requirement of Bx42 for eye growth and development was observed using the GMR-GAL4 and *ey*-GAL4 driver lines, as the results indicated that adults of GMR-GAL4/UAS Bx42-SNW flies have small and rough eyes, and the adults of ey-GAL4/UAS Bx42-SNW flies have a highly reduced eye size or lack most of the eye. As mentioned before, in contrast to GMR-GAL4/UAS Bx42-SNW flies, the ey-GAL4/Bx42-SNW flies showed variant adult eye size which would interfere with my planned modifier screen. Therefore, GMR-GAL4/UAS Bx42-SNW was selected for the following experiments.

In contrast to GMR-GAL4/UAS Bx42-SNW flies, GMR-GAL4/UAS Bx42-FL flies showed a WT eyes, indicating that an increase in the Bx42 dosage does not disrupt eye development. The dominant effect observed (see below) was caused by the expression of Bx42-SNW and it was dosage dependent an ideal requirement for my planned modifier screen for Bx42 interactors.

4.1.1.2 Evidence that Bx42-SNW is dominant negative involved in Notch pathway

The eye phenotype caused by overexpression of a single dominant allele Bx42-SNW using GMR-Gal4 is similar to that obtained by Dr. Negeri, by induction of Bx42 RNAi (Negeri., 2003), and therefore a loss of function phenotype (dominant negative). Moreover, it is comparable with eye phenotypes which arise when the *Notch* signaling pathway is blocked or weakened. For instance the *ey*-Gal4 induced overexpression of a dominant negative form of Notch ECN, resulted in the formation of reduced eyes (Kurata et. al, 2000). Similarly, overexpression of the Notch antagonist Hairless in the eye imaginal discs leads to greatly reduced eyes, and in extreme cases, the deletion of the eyes (Go et al., 1998; Müller et al., 2005). Additionally, Ectopic expression of Hairless enhances the Bx42-SNW phenotype and N-IC overexpression suppresses the Bx42-SNW eye phenotype (Negeri unpublished data). These data also show that an agonistic relationship between Bx42 and the proteins of the Notch signaling pathway, Notch and Mastermind and an antagonistic relationship with the Notch repressor Hairless. Bx42 may play a role as a coactivator of Notch since the expression of the dominant negative allele of Bx42 has a comparable effect on the eye development, as inhibition of the Notch pathway.

4.1.2 Bx42-SNW induced reduction in eye size corresponds with a block of mitosis and elevated apoptosis

To characterize the role of Bx42 on cellular proliferation in *Drosophila* eye imaginal discs, I compared S-phase cells by EdU incorporation and G2/ M-phase cells using the anti-pH3 antibody between Bx42-SNW animals and wild type controls. The EdU incorporation experiment showed no differences between *GMR-GAL4/ UAS-Bx42-SNW* and the controls *GMR-GAL4* and *UAS-Bx42-SNW*. In contrary, the number of mitotic cells identified by phosphohistone H3 (PH3) staining in the second mitotic wave was highly reduced. These results indicate that Bx42-deficient cells are compromised in their ability to progress through the cell cycle. They can go through G1/S but not in G2/M. Interestingly, removal of Notch in the eye caused a cell autonomous block of mitosis as assayed by phosphohistone H3 (PH3) staining, and clones of cells mutant for the Notch ligand Delta showed the same results (Baonza & Freeman., 2005). A significant decrease in proliferation was also observed in *Drosophila's* pupal myogenic progenitors when Notch function was ablated by RNAi, as measured by the mitotic marker phosphorylated histone H (Krejcí et al., 2009). Such a defect may be expected to lead to mitotic problems and the elimination of the affected cells by apoptosis to avoid the accumulation of damaged or dysfunctional cells. I examined whether the dominant negative

Bx42-SNW might have such an effect in vivo. When *UAS Bx42-SNW* was overexpressed under the control of the GMR-GAL4 driver, a large number of cells in the affected area (the posterior part of the eye disc) underwent apoptosis, showed using immunostaining with activated caspase-3 antibody and cell death marker acridin orange staining. These results contrast the results in our group, which reported just a slight increase in the number of apoptotic cells in *GMR-GAL4/ UAS Bx42-SNW* eye imaginal discs, which were explained as an effect of the driver line *GMR-GAL4* itself (Lehman, 2006; Negeri unpublished). The difference between the results may be due to the experiment conditions such as the temperature, since the flies cross used in this work were incubated at 25 ºC, and in previous experiments they were incubated it at room temperature. Interestingly, overexpression of the Notch antagonist Hairless (H) also increases the rate of cell death in imaginal tissues. Hairless (H) is a major antagonist of N signaling and increasing H activity is inversely correlated with N activity. Müller and his coworkers observed that overexpression of H results in reduction of tissue size, and this is accompanied by pronounced increase of apoptotic cells, which are restricted to the area where ectopic H expression is induced in third instar larval discs (Müller et al, 2005).

4.1.3 Potential modulators of Bx42 mediated cellular proliferation in *Drosophila* eye

On the basis of my findings a genetic based screen to identify genes involved in cell cycle regulation that modify the Bx42-SNW eye size phenotype was undertaken. A loss- and gain-of function screen was performed with ~70 candidate alleles, and eight lines were isolated as modifiers of the Bx42-SNW small eye phenotype. These genes could be grouped in two classes, signaling pathway genes and the cell cycle regulators.

Signaling pathway genes:

Drosophila eye development is a complex process involving inputs from multiple signal transduction pathways including EGFR, Hh, Notch, Dpp and Wingless signaling. Therefore, it was not surprising, to identify signaling molecules as modulators of Bx42-SNW small eye phenotype.

EGFR: In *Drosophila* developing eye, the epidermal growth factor receptor EGFR plays two distinct roles, it prevent cell cycle entry and keeps four types of photoreceptor neuron (R2-R5) from re-entering the cell cycle, and promotes G2/M progression of other cells during the SMW. Additionally, it was reported to play other roles, early in development, it is needed at the disc margins. It regulates *rough* expression in the furrow, thereby affecting the spacing of R8 cells,

and it is needed to promote survival of cells once the differentiation program has begun behind the furrow (Dominguez et al., 1998; Baker and Yu, 2001; Baonza et al., 2002; Yang & Baker, 2003; Firth& Baker, 2005; Baonza& Freeman, 2005). As reported before, antagonism between *EGFR* and *N* signaling pathways maintains the distinction between cell cycle progression and differentiation in this context. The EGFR activity that promotes fate specification also antagonizes Notch to prevent cell cycle entry, whereas Notch antagonizes cell fate specification and promotes cell cycle entry (Firth& Baker, 2005; Yang & Baker, 2006). In this work, it was found that a dominant negative form of EGFR enhances the phenotype caused by Bx42-SNW. This effect could be explained as an additive effect, since the expression a dominant negative form of EGFR using GMR-GAL4 itself resulted in small eyes. However, overexpression of EGFR in a GMR-GAL4/+; UAS Bx42-SNW/+ background was found to suppress the Bx42-SNW small eye phenotype. This may be explained through EGFR´s role in promoting G2/M transition. Moreover, such genetic interaction between a member of Notch signaling and EGFR was reported before by Müller and colleagues, they identified several EGFR-pathway members as modifiers of H, in their genetic modifier screen on Hairless gain-of-function phenotype (Müller et al., 2005). Kankel and his coworkers, in a screen for Notch signaling modifiers using the Notch transcriptional co-activator mastermind, also detected a crosstalk between Notch and EGFR signaling through the transcription factor *klumpfuss* (*klu*) (Kankel et al., 2007).

Dpp: The *Drosophila* TGF-β homolog *decapentaplegic* (*dpp*) participates in the growth and patterning of many tissues in *Drosophila*. In *Drosophila* eye imaginal disc Dpp signaling is essential for MF initiation (Curtiss and Mlodzik., 2000). Dpp acts also to induce G1 arrest in the anterior part of the morphogenetic furrow through the downregulation of Cyclin E levels and E2F activity (Horsfield et al. 1998; Escudero & Freeman, 2007). Consistent with that, overexpression of *dpp* in the eye imaginal disc reduces the number of cells in S phase (Horsfield et al. 1998). In my screen, UAS-Dpp was identified as a strong enhancer of the small-eye phenotype caused by Bx42-SNW. Notch and Dpp signaling crosstalk was previously reported, Müller and colleagues (2005) found that overexpression of Dpp decreased eye size of GMR driven Hairless expression. Besides, it was reported before that *Drosophila* Bx42 is implicated in Dpp signaling pathway (Negeri et al., 2002; El Hachoumi, 2007). It seems that Bx42 is partially independent of Dpp roles in eye imaginal discs, because overexpression of Dpp couldn't rescue the Bx42-SNW small eye phenotype. It will be interesting in the future to analyze this interaction in more details, by identification of the changes in S- and M- phase cell in the second mitotic wave.

Armadillo: armadillo is a transducer of wingless signaling. Wg inhibits eye specification and patterns the dorsal-ventral axis in the eye disc (Legent & Treisman., 2008). Increasing the levels of Armadillo in the wild-type eye (*ey>arm*) resulted in small eyes (Singh et al., 2006), and expression of activated *arm* in clones of cells in eye disc resulted in a block of photoreceptor differentiation within the clone (Hazelett et al., 1998). In this work it was found that overexpression of armadillo enhances the Bx42-SNW small eye phenotype, that seems to be an additive effect since armadillo alone resulted in a small eye phenotype. On the other hand, the interaction between Notch and Wingless signaling is also often reported (reviewed by Martinez Arias., 1998). Additionally, activation of Wg pathway by overexpression either of the Wg or of downstream components such as Armadillo decreased eye size of GMR driven Hairless expression (Müller et al., 2005).

The cell cycle regulator genes:

E2F/DP:

E2F/Dp transcription factors (made up of an E2F subunit and its dimerization partner) play key roles in proliferation, since they target many cell cycle regulating factors and other genes responsible for cell differentiation and apoptosis (Van den Heuvel & Dyson. 2008). In the *Drosophila* eye imaginal disc, E2F is expressed in proliferating cells and in post-mitotic cells, it is required for cell proliferation, and E2F-deficient cells have abnormal morphology and poor survival (Brook et al., 1996). Additionally, previous studies showed that, flies carrying two copies of both GMR DP and GMR E2F had severely abnormal eyes. These eyes were rough in appearance, ommatidia lacked their regular hexagonal shape and many more bristles were apparent, but flies carrying one copy of GMR DP and GMR E2F showed only minor defects (Du et al., 1996). Moreover, these authors reported, that overexpression of E2F and DP leads to ectopic S phase cells posterior to the furrow and allows neuronal cells to re-enter the cell cycle and increased cell death (Du et al., 1996). Additionally, Davidson& Duroni (2012) reported that that hyper-accumulation of E2F1 during S phase represents a form of proliferative stress during development that is sensed by the apoptotic machinery and results in the elimination of cells with excess E2F1 activity in order to ensure normal development of rapidly proliferating tissues like *Drosophila* eye disc. On the other hand, as mentioned in the introduction, Notch is required in *Drosophila* eye imaginal disc to overcome the G1 cell cycle arrest in the morphogenetic furrow (MF) to trigger the initiation of the second mitotic wave SMW. Notch triggers the onset of proliferation by multiple pathways, including the activation of E2F. Delta to Notch signaling downregulates the inhibition of E2F1 by Rb (Baonza & Freeman, 2005; reviewed in Baker, 2007; Carthew, 2007). In my study, UAS E2F/Dp was

identified as a strong enhancer of Bx42-SNW small eye phenotype with a high percentage of pupal lethality.

Actually, the overexpression of E2F in a Bx42-SNW background is expected to rescue the small eye phenotype. In contrary to the expectations, it enhanced the phenotype. On the other hand, maybe the weakened of Notch signaling because of the overexpression of the dominant negative Bx42-SNW resulted in upregulation the inhibition of E2F by Rb. Another explanation may be that Bx42-SNW potentiates the effect of overexpression of E2F/Dp through increasing the number of S-phase cells, inducing proliferative stress and thus raises the number of apoptotic cells.

Rb:

The retinoblastoma protein Rb plays important roles in many processes implicated in cell fate decisions, including cell cycle, differentiation and apoptosis. In cell cycle regulation, Rb interacts principally with the E2F transcription factor family members to inhibit the transcription of many genes controlling cell cycle progression (reviewed by Van den Heuvel and Dyson., 2008; Gordon & Du, 2011). In my study, a genetic interaction between Rb and Bx42 was demonstrated, as the Rb-RNAi and *Rb* mutants could enhance the Bx42-SNW induced small eye phenotype. SKIP (the human homolog of Bx42) interacts with the oncogene Ski (Prathapam et al., 2001a). Ski was first shown to interact with Rb and to inhibit transcriptional repression mediated by Rb (Tokitou et al., 1999). Later, Prathapam and colleagues (2002) could show that SKIP interacts with Rb, and in cooperation with Ski, it can overcome Rb-induced transcriptional repression. A protein-protein interaction between SKIP and Rb was also demonstrated by these authors. The interaction between Ski and SKIP is evolutionary conserved since it was also demonstrated in *Drosophila*. El Hachoumi could show that Bx42 interacts with dSno, the *Drosophila* homolog of human Ski/ Sno proto-oncogene family in the yeast two hybrid assay and also physically interacts as shown by in vitro pull down (El Hachoumi, 2007). The genetic interaction indicates that, Bx42 may affect the cell cycle partially through its interaction with Rb. It will be interesting in the future to study if the protein-protein interaction between Bx42 and Rb is also conserved. If so, that will provide an additional explanation for the results obtained here.

On other hand, similar to the proposed UAS E2F/Dp effect mentioned before, an explanation of enhancement of the Bx42 small eye phenotype induced by Rb RNAi or mutants may be that the overactivation of E2F resulted from Rb downregulation increases the number of S-phase cells and by induction of proliferative stress a number of cells might initiate apoptosis. More experiments are required to substantiate this hypothesis.

UAS Cyclin B3; UAS Cdk1; *tribbles* mutant:

Progress through the cell cycle can be regulated at G0/G1, G1/S, and G2/M transitions. At each of these points, the activity of a Cyclin-dependent kinase (Cdk) is required to proceed through the transition. Cdk activity is, in turn, dependent upon the presence of the appropriate Cyclin (the regulatory subunit in all Cyclin/Cdk heterodimers) and activating phosphorylation, and on the other hand on the absence of Cdk inhibitors and inhibitory phosphorylation. While Cyclin alternates between synthesis and degradation, constant levels of the Cdks are maintained during the cell cycle. The Cyclin dependent kinase Cdk1 is maintained in an inactive hyperphosphorylated state during G1, S and G2 stages. For the G2/M transition, both mitotic Cyclins and a phosphatase of the CDC25 family are required. In addition to the control at transition points, a significant feature of the cell cycle is that it cannot go backward. This directionality appears at least in part to be imposed by an irreversible biochemical step, the proteolysis of key molecules. Many components of the cell cycle are subject to rapid and/or regulated degradation via the ubiquitin-proteasome pathway. This may also ensure that the cycle is sufficiently responsive to its regulatory cues (Mata at al., 2000; Morgan, 2007). In this screen, three genes involved in G2/M transition namely *Cyclin B3, cdk1* and *tribbles*, were identified as modifiers of the Bx42-SNW small eye phenotype. Cyclin B3 is present in addition to Cyclins A and B in mitotically proliferating cells and not detectable in endoreplicating tissues of *Drosophila* embryos. Cyclin B3 is coimmunoprecipitated with Cdk1 (the G2/M Cdk) but not with Cdk2 (the G1/S Cdk). It is degraded abruptly during mitosis like Cyclins A and B. Genetic analyses indicate functional redundancies. Double and triple mutant analyses demonstrate that Cyclins A, B, and B3 cooperate to regulate mitosis (Jacobs et al., 1998). In addition, Gallant and Nigg (1994) demonstrated that Human Cyclin B3 might share functional properties with both A and B-type cyclins. In my screen I found that over expression of Cyclin B3 suppressed the Bx42-SNW small eye phenotype. On the other hand, overexpression of the mitotic Cyclin-dependent kinase Cdk1, which is the catalytic subunit of the mitotic cyclins A, B, and B3, also robustly rescued the small eye phenotype induced by Bx42-SNW. Cdk1 function is regulated also by inhibitory phosphorylation by Wee1 (in *Drosophila*: Dwee1) and dephosphorylation by Cdc25 (in *Drosophila*: String) (Morgan, 2007). Additionally, many cell -cycle regulators are destroyed by ubiquitin-dependent proteolysis. In *Drosophila*, the *tribbles* (*trbl*) gene encodes a mitotic inhibitor that promotes the degradation of String/CDC25 protein via the proteasome pathway (Mata et al. 2000). Genetically, Tribbles was also identified as Bx42 interaction partner, since a *tribbles* mutant allele could rescue the small eye phenotype of Bx42-SNW.

Interestingly, most of Bx42-SNW genetic interaction partners that enhanced the small eye phenotype are factors which potentially affect the cells to accumulate in S-phase (UAS-E2F/Dp, Rb RNAi, EGFR mutant) and the factors which rescue the small eye phenotype are factors that contribute in the G2/M transition (Tribbles mutant, UAS Cdk1, UAS Cyclin B3, UAS EGFR). In other words, it seems to be that a Bx42 loss of function led the proliferating cells to accumulate in S-phase and that Bx42 is required to progress through S-phase to G2 in the SMW of *Drosophila* eye imaginal disc. These results also in fit with the observations made with cell cycle markers in GMR-GAL4/UAS-Bx42-SNW eye discs, which indicate that the cells in the second mitotic wave enter S-phase (EdU incorporation) but don't undergo M-phase (no H3S10-phosphohistone staining).

4.1.4 Expression of genes involved in cell cycle regulation is reduced in eye discs of GMR4/ Bx42-SNW animals

In the developing eye disc, 8-9 rows of cells become arrested in G1 phase for about 14 hours within (and anterior to) the MF prior to differentiation. Immediately posterior to the MF, a subset of these G1 phase cells is induced by patterning mechanisms to terminally differentiate into ommatidial precluster cells, while the remaining cells synchronously enter S phase (Wolff and Ready, 1993). In wild type, Cyc E protein is present in a subset of the asynchronously proliferating cells. It is also present in a band of cells immediately posterior to the MF, corresponding to S phase cells (Richardson et al., 1995). Within these zones, there appeared to be a greater degree of overlap between Cyclin A- and Cyclin E- than between Cyclin B and Cyclin E (Thomas et al., 1997; Horsfield et al.,1998) , this suggests that Cyclin A is expressed earlier in the cell cycle than Cyclin B (reviewed by Follette and O'Farrell, 1997). Cyclin A has been implicated in S phase control but its role in promoting DNA replication is less clear than that of Cyclin E. There is evidence from *Drosophila* that Cyclin-A-associated kinase activity can induce DNA replication. For example, ectopic Cyclin A expression in the eye imaginal disc - resulting from the *roughex* mutation - leads to ectopic S phase (Thomas et al., 1994). However, *Drosophila* embryonic endocycles still occur in the absence of Cyclin A (Knoblich et al., 1994), indicating that Cyclin A is not always essentially required for replication. One possible resolution of this apparent contradiction, suggested by Follette and O'Farrell (1997), is that Cyclin A may be required for the completion of replication specifically in mitotic cells with a G1 phase. In these cells, Cyclin E is downregulated prior to the completion of DNA replication, in preparation for the next G1 phase. In agreement with that, Sallé and colleagues (2012) could show that CycA proteins accumulate during the final period of endoreplication in the *Drosophila* bristle lineage. Once replication is complete, Cyclin A plays a negative role in

controlling DNA replication, as it is required during G2 phase to prevent replication of the genome (Follette and O'Farrell, 1997). Additionally, in wild type eye-imaginal discs, Dacapo expression is activated by EGFR and Hedgehog (Hh) signaling in post-mitotic cells in and posterior to the MF (Wolff and Ready, 1991; Lane et al. 1996; Firth and Baker 2005; Escudero and Freeman 2007; Sokhanova et al., 2007; Baig et al., 2010).

Since I found that Bx42 plays an important role in cell cycle progression, I investigated the expression of regulators of cell cycle control in a Bx42-SNW background, and I found that Cyclin E protein levels were not changed. *Dacapo* transcript levels also were not affected. In contrary to Cyclin E, the proteins levels of mitotic cyclins A and B were strongly reduced in the second mitotic wave of GMR-GAL4/UAS Bx42-SNW eye imaginal discs. Most of the data indicate that the observed Bx42-SNW phenotype depends on the role of Bx42 in Notch signaling, in particular, because Notch overexpression suppresses the Bx42-SNW small eye phenotype (Negeri, unpublished data). Additionally, removal of Notch in the eye, by generating clones of cells homozygotes for a null allele of Notch or the Notch ligand Delta, caused a cell-autonomous block of mitosis, as assayed by phosphohistone H3 (PH3) staining, *Notch* mutant cells also failed to express Cyclin B, which accumulates in G2 and breaks down at mitosis. Moreover, Cyclin A was found to be significantly downregulated in these cells. However, cells in *Notch* mutant clones expressed Cyclin E, and there was no upregulation of Dacapo (Baonza & Freeman., 2005). Cyclin E accumulation also occurred in *Su (H)* mutant cells, showing that Cyclin E was not induced by Notch (Firth and Baker, 2005). The data of Reynolds- Kenneally & Mlodzik (2005) also support a role for Notch signaling in cell cycle progression at the SMW. Cells expressing N^{act} within the morphogenetic furrow and posterior to it incorporated BrdU cell-autonomously, indicating that N^{act} pushes cells into S-phase and prevents cells from entering G1 arrest. Accordingly, the pattern of BrdU incorporation in the SMW was extended, which suggests that more cells enter S-phase. This was also evident with other cell cycle markers, e.g. H3S10-phosphohistones. Similarly, expression of the G2 marker, Cyclin B, is upregulated in N^{act} clones throughout the eye field. Overexpression of Bx42 -SNW in eye imaginal discs does not affect the G1-S transition in the SMW, since the cells normally incorporated the S- phase marker EdU. However, accurate counting of these cells could not be done, because of difficulties by overlapping cell staining (this aspect will be considered in the second part of my results, using *Drosophila* S2 cells as an experimental system). On other hand, my results imply that Bx42 is not required for the Dpp-dependent G1 arrest in the SMW, because Cyclin E protein levels which are regulated by Dpp were not changed. Additionally,

UAS-dpp could not rescue the Bx42-SNW small eye phenotype; in contrary, it was a strong enhancer.

4.2 Investigating the role of SKIP/Bx42 in *Drosophila* S2 cells

Genetic studies on *Drosophila* S2 cells have proven powerful in identifying the cell cycle machinery and its regulatory mechanisms. In recent years, RNAi has been used in a variety of genome-wide screens and single molecule studies to elucidate the mechanisms of cell cycle progression (Bettencourt-Dias & Goshima., 2009; Mohr et al., 2014). Therefore, to determine whether Bx42 plays a role in the proliferation of S2 cells, I knocked down the expression of Bx42 in S2 cells using dsRNA. In comparison to dsOFP treated controls the function of Bx42 in cell cycle progression could be followed by a variety of assays, including immunostaining, semi q-RT PCR, western blot and FACS analysis.

4.2.1 RNAi knockdown of Bx42 results in S2 cells results in cell cycle arrest

To determine the most efficient strategy of Bx42 knockdown in *Drosophila* S2 cells, I carried out RNAi in S2 cells against two different regions of the protein (400bp and 700bp templates). Almost identical results were obtained for both targeting dsRNAs. That is in accordance with the work of (Betz, 2003) which concluded that shorter dsRNAs appear be as effective as longer dsRNAs in inducing RNA interference in *Drosophila* S2 cells in the range between 180-778bp. During optimization of dsRNA experiment, three different concentrations Bx42-dsRNA (8, 12, 16 µg/3ml) were used. Knockdown was most successful at the highest concentration (16µg/ 3ml), resulting in a nearly non-proliferative/ cell cycle arrest state. Therefore, this was the concentration used for all experiments. This result indicates that Bx42 is essential in cell proliferation processes. Notably, the size and shape of most dsRNA-Bx42 transfected cells were not changed, but the cells lose the ability to form colonies, as the healthy S2 cells tend to proliferate and form colonies. To be sure that the non-proliferating state is due to Bx42 depletion, I detected the levels of both Bx42 protein and RNA and could show that they are significantly reduced in these cells in comparison with control. Additionally, semi qRT-PCR showed that, the amounts of Bx42 RNA decreased 24h after addition of Bx42-dsRNA, and are reduced to (under my PCR conditions) undetectable levels 48h posttransfection. This early reduction of RNA was also reported for other proteins. Clemens and coworkers could detect DSH3PX1 protein reduction as early as day 1 dsRNA DSH3PX1 posttransfection in *Drosophila* S2 cells (Clemens et al., 2000). Similarly, March and Bentley could detect the reduction of Cyclin E RNA at 24h dsRNA Cyclin E posttransfection (March and Bentley, 2007).

4.2.2 Bx42 depletion slows cell proliferation and increases the proportion of cells in S-phase

Because loss of cell growth could arise from loss of cell proliferation or from cell death, several experiments were performed to differentiate between these possibilities. Trypan blue staining was used to examine the cell viability and it was found that most of the cells were still viable 96h posttransfection. According to this result, I hypothesized that the reduction in the number of dsRNA Bx42 transfected cells might correlate with cell proliferation. Therefore, cells undergoing S-phase and M-phase were scored. The data showed a strong decrease in the number both of S-phase cells and M-phase at 96 posttransfection. These data suggest that the cells were arrested during this period. Therefore, S-phase cell number was determined at 24 and 48h posttransfection and the results showed that the population of S-phase dsRNA-Bx42 transfected cells was increased compared to the dsRNA-OFP treated controls. In contrast, the mitotic cell number of dsRNA Bx42 transfected was slightly less than that of the control on 24h posttransfection. Although the differences between both conditions were still small at this time point, the data suggest that the dsRNA Bx42 transfected cells tend to accumulate in S-phase and have a problem to go through G2/M. In other words, it means that these cells are arrested in either S- or G2-phase. These results in agreement with the results of Somma and colleagues (2008) which identified in their RNAi screen dsBx42 out 13 dsRNAs that result in the absence of dividing cells at 96h after initiation of treatment. Additionally, tubulin staining in the my work showed no defects in the mitotic cells spindles of S2 dsRNA Bx42 transfected cells at 96h posttransfection. That is in contrast to the study of Eggert and colleagues (2004), which reported that dsBx42 in *Drosophila* Kc cells led to defect cytokinesis, binucleate cells with microtubule extensions. However, such phenotypes may also result from incomplete replication and failures in spindle checkpoint regulation that results in aberrant mitosis in cells with a functional spindle. Moreover, *Drosophila* S2 cells and Kc cells are two different cell types that may behave somewhat different (Neal et al. 2003; Cherbas et al., 2011) and the assay conditions also was slightly different. Interestingly, the observed cell cycle phenotype is similar to the effects of SNW1/SKIP depletion in human cells. SNW1/SKIP depletion in HeLa resulted of mitotic arrest and cytokinesis defect (Kittler et al,2004; Sato et al., 2015), but Sato and colleagues (2015) reported that neither MDA-MB-231cells nor MCF7 cells showed mitotic arrest and concluded that the phenotype induced by dsRNA Bx42 may be dependent on cell type.

To investigate further the dsRNA-Bx42-induced slowing of cell division, flow cytometry was used to examine the cell-cycle profiles of control and dsRNA-Bx42 treated S2 cells. By analyzing these profiles, it can be concluded that, there was a general decrease in G1

and G2 cells and an increase in S-phase cells with increasing time of Bx42 knock down. This increase in the fraction of cells in S phase upon Bx42 depletion could be as a result of increased entry of the cells into S phase (the cells are forced to enter S-phase) or a result of the activation of the intra-S phase checkpoint (the cells have a problem to pass through S- phase to G2/M). This might be analyzed by comparing the expression and the activation state of checkpoint kinases at different time points following induction of Bx42- and OFP-control-RNAi. To explore the possible mechanism relevant to Bx42 RNAi dependent down-regulation of cell proliferation, western blot analysis and semi qRT-PCR experiments were performed to follow the changes of the cell cycle regulators over time. On the other hand, the non-proliferating cells might undergo one of different fates: apoptosis, senescence, or quiescence. Therefore, experiments were done to define Bx42 depleted cells underwent one of these fates.

4.2.3 Bx42 RNAi results in downregulation of cell cycle regulators

To gain insights into the biological role of Bx42, semi qRT-PCR analysis of key cell cycle regulatory molecules was performed following the dsRNA-mediated knockdown of Bx42 in S2 cells. I found that dsRNA Bx42 correlated with decreased transcription of *E2F*, *cyclin A*, *dacapo* and *cyclin B*, whereas no changes in transcripts levels were detected for *Retinoblastoma*, *String*, *Cdk1*, and *Cyclin E*. Additionally, Western Blot analysis confirmed the depletion of Cyclin A and B proteins 96h posttransfection. These results are consistent with the results of the first part of my work, when I showed that the expression of the dominant negative allele of Bx42 in the posterior part of eye imaginal disc resulted in downregulation of Cyclin A and Cyclin B posterior to the MF. Similar results were obtained in human cells by Liu and colleagues (2014) which found that SKIP siRNA downregulated the protein levels of Cyclin A and Cyclin B in human breast cancer cell lines MCF-7 and MDA-MB-231.

In contrast, Dacapo RNA level was not changed in GMR-GAL4/UAS Bx42-SNW flies, in comparison with GMR-GAL4. However, this result does not exclude the possibility that Bx42 is implicated in the transcription or splicing of Dacapo in *Drosophila* eye, because Dacapo becomes expressed in the morphogenetic furrow outside and anterior to the region in which Bx42-SNW was induced (Figure 3-16). It only shows that Dacapo was not overexpressed in the second mitotic wave of GMR-Gal4/UAS Bx42-SNW eye discs. On other hand, the EdU incorporation pattern in *ato*-GAL4/UAS-Bx42-SNW eye discs provides indirect evidence that Bx42-SNW expression at the MF does not affect Dacapo expression, since no excessive EdU incorporation was discovered (Figure 3-4, A, A´). Notably, SKIP the human homolog of Bx42,

was found to be required for the basal and stress-induced expression of *p21*, the human homolog of Dacapo in two human cancer cell lines (Chen et al., 2011).

4.2.4 The cell fate of Bx42 transfected cells

4.2.4.1 Following Bx42 RNAi knockdown cell viability is slightly decreased.

Since Trypan Blue is not an apoptosis marker I initiated additional tests programmed cell death of S2 RNAi treated cells. Activation of the caspase-3 pathway is a hallmark of apoptosis and can be used in cellular viability assays to quantify programmed cell death. Caspase-3 is associated with the initiation of the "death cascade" and is therefore an important marker of the cell's entry point into the apoptotic signaling pathway. In the present work protein and RNA levels of the apoptotic factor caspase-3 were detected by immunostaining and semi q-RT-PCR. The RT-PCR showed no differences among control and Bx42 depleted cells samples. The immune staining results showed that about ~92% of the dsRNA Bx42 treated cells were not stained with cleaved caspase-3 antibody 96h post transfection. Although this was a small increase compared to dsRNA-OFP controls it was probably not functionally significant.

4.2.4.2 Bx42 transfected cells do not undergo senescence.

Cellular senescence refers to the essentially irreversible arrest of cell proliferation. Senescent cells remain metabolically active but display characteristic changes in cell morphology and gene expression and typically upregulate senescence-associated markers like β-galactosidase (SA-β-gal) activity (Dimri et al., 1995; Shelton et al., 1999) or a distinct heterochromatic bodies called "senescence-associated heterochromatic foci" (SAHF) (Narita et al., 2003). Bx42-S2 transfected cells neither showed changes in cell morphology, nor in nuclear morphology, and they did not develop the senescence-associated heterochromatin foci (SAHF). In addition, the proportion senescence-associated ß-galactosidase stained cells did not significantly in dsRNA Bx42 transfected cells in comparison to the controls. Rb and p53 are typically activated during senescence, and enforced expression of either protein induces senescence in some cell types (Ferbeyre et al., 2002; Lee et al., 2000; Blomen & Boonstra, 2007). In my work, semi qRT-PCR showed no differences in the levels of p53 transcripts between dsRNA-Bx42 transfected cells and the control cells. However, the immune staining using an antibody against P53 showed a small increase in P53 positive cells in Bx42 transfected cells comparable with control cells. These P53 positive cells might be cells undergoing apoptosis, as P53 is also implicated in apoptosis and the ratio of P53 positive cells is similar to the ratio of apoptotic cells mentioned before. Additionally, Rb protein and RNA levels were not changed. More recently, Dacapo was

proved also to be involved in *Drosophila* senescence (Nakamura et al., 2014), and it was found that Dacapo/p21 contributes to progenitor cell quiescence of lymph gland cells (Kalamarz et al., 2012). In my study, I found that Dacapo RNA level is strongly reduced and undetectable 48h post transfection.

In summary, my results of the second part indicate that, besides some apoptotic or senescent cells, the most of Bx42 RNAi knockdown cells seem to enter a prolonged S-phase because of the absence of the essential cell cycle factors Cyclin A and B and downregulation of the transcription factor E2F. In addition, these cells also are forced into S-phase because of down regulation of the G1/S inhibitor Dacapo.

4. Conclusion

In my study, I found that the expression of Bx42-SNW, a truncated dominant negative version of Bx42 in eye imaginal discs resulted in small and rough eyes. Analyzing the cell cycle in the affected discs showed no significant differences in the number of S-phase cells, but a strong reduction of mitotic cells and the downregulation of Cyc A and Cyc B in the normally mitotic active SMW region of the eye disc. Dacapo expression was not changed in these experiments since Dacapo is expressed in MF anterior to the expression domain of the GMR-GAL4 driver line. These results are similar to the results of previous Notch signaling studies, which indicated that downregulation of Notch members in *Drosophila* eye resulted in downregulation of Cyc A and Cyc B but not Cyc E and reduction of M-cells. Additionally, Notch overexpression can rescue the Bx42-SNW small eye phenotype. These results suggest that Bx42 is required for cell cycle control in the *Drosophila* eye through its role in Notch signaling. In addition, Bx42-SNW overexpression resulted in elevated apoptosis in the *Drosophila* eye imaginal disc, probably to avoid disruption of further development by accumulation of supernumerary non-dividing cells. This study aimed also at finding genetic interactors of Bx42 as factors that modify the small eye phenotype resulting from overexpression of Bx42-SNW in the eye. The modifiers identified were E2F/Dp, Rb, Tribbles, Cdk1, Cyclin B3, EGFR, Dpp and Armadillo.

In the second part of my thesis I performed RNAi knockdown of the Bx42 gene in S2 cells. In contrast to the control knockdown by dsRNA-OFP, Bx42 knockdown resulted in cell cycle arrest. The results indicate that, besides some apoptotic or senescent cells, the most of Bx42 RNAi knockdown cells seem to enter a prolonged S-phase because of the absence of the essential cell cycle factors Cyclin A and B and downregulation of the transcription factor E2F. Additionally, contribution of Dacapo to the arrested state became evident following Bx42 knockdown in S2 cells. In the absence of Bx42 Dacapo transcription was dramatically reduced in S2 cells, suggesting that these cells also are forced into S-phase because of down regulation of the G1/S inhibitor Dacapo.

Taken together, these findings indicate that Bx42 might contribute to cell proliferation through tissue specific control of cell cycle regulators. In eye imaginal disc, Bx42 is required for Cyclin A, and Cyclin B expression and expression of the dominant negative allele Bx42-SNW resulted in downregulation of cell cycle regulators Cyclin A and Cyclin B, but not Cyclin E. That seemed to be through its role as notch transcription co-factor. Additionally, E2F-Dp and Rb seemed to be included in Bx42-SNW small eye phenotype. Similarly, Bx42 is also required in S2 cells for

the expression of Cyclin A, Cyclin B, E2F and Dacapo. If these effects depend on its role in Notch pathway or independent is still an open question (Figure 4-1).

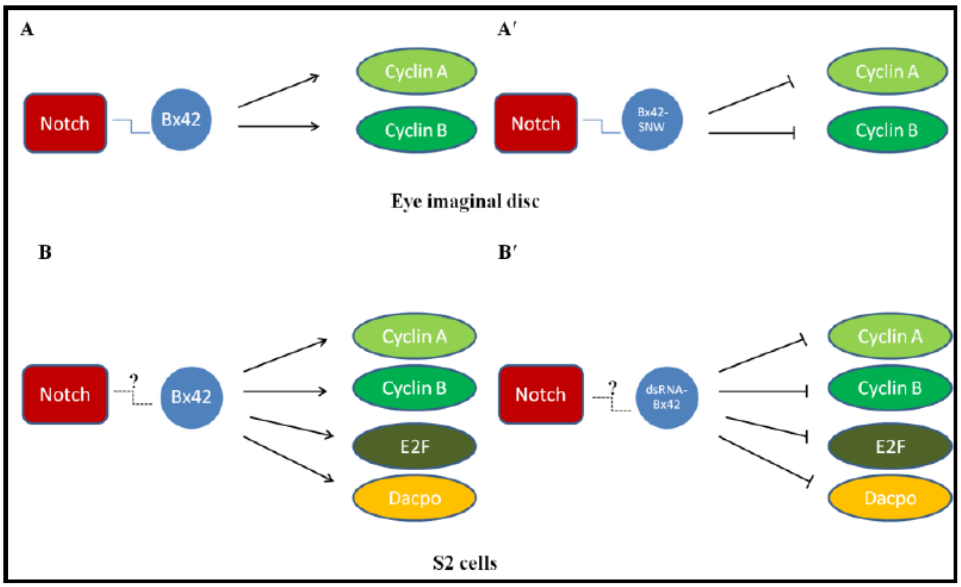


Figure 4-1: Schematic representation of the role of Bx42 in cell cycle.
(A) In eye imaginal disc, Bx42 is required for Cyclin A, and Cyclin B expression, that seemed to be through its role as notch transcription co-factor. (A′) Expression of the dominant negative allele Bx42-SNW in eye imaginal disc resulted of downregulation of Cyclin A and Cyclin B. (B) In S2 cells Bx42 is required for the expression of Cyclin A, Cyclin B, E2F, and Dap, it is not yet defined if that's through its role in Notch pathway. (B′) Bx42 depletion causes downregulation of each of Cyclin A, Cyclin B, E2F, and Dap in S2 cells.

My results also raise a number of questions that ask for continuation of experiments

1. How are the observed Bx42effects mediated? Does the genetic interaction indicate a direct interaction in the expression (transcription or splicing)? Might be there interleaving factors that did not yet become apparent by my analysis? A clonal analysis using loss of function alleles of Bx42 might be a means to approach this question.
2. In case of suggested transcriptional controls: is Bx42 bound at regulatory sites of the suggested target genes? (analysis by ChIP)
3. Alternatively, physical interactions between Bx42 protein and the proteins encoded by interacting genes could be tested. In the light of demonstrated genetic interaction between Bx42 and Rb, and the known protein-protein interaction between the human Bx42 homolog SKIP and Rb, it will be interesting to study if this protein-protein interaction is also conserved in *Drosophila* and how this bears to the Bx42 function in this case.
4. More experiments needed to determine if Bx42 plays its role in proliferation control as a Notch transcription cofactor or as a splicing factor, or both as the human SKIP, which was proposed to play a dual role in VDR-mediated gene expression (Zhang et al., 2003).

5. Is Bx42 function in S2 cells also mediated by signaling? *Notch* expression data in DRSC (*Drosophila* RNAi Screening Center) showed that *Notch* may be expressed in S2 cells; hence the proposition that Bx42 affect cell cycle in S2 cells through its role in Notch signaling is not excluded. But is the Notch pathway also activated in S2 cells by the Delta or Serrate ligands? Future work might provide a confirmation. On contrary, gene expression data of *EGFR* and *Dpp* in S2 cells showed a low expression of both genes, which suggests that Bx42 role in S2 proliferation is likely to be independent of these two pathways.

Caveat: It is important to mention that the unique structure of Bx42 suggested by Yan and colleagues (2015) may facilitate its interaction with a large number of other proteins and may enable its participation in different biological processes and at the same time may complicate our analyses.

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6 Appendix

The results of the genetic screen.
S: suppressor of Bx42-SNW small eye phenotype, E: enhancer of Bx42-SNW small eye phenotype, N indicates the absence of interaction.

Cell cycle control system			
Stock number	Gene	Mutant	Result
6642	*Cdk1, (cdc2)*	UAS Cdk1	N
6641	*Cdk1, (cdc2)*	Cdk1 loss of function	N
6636	*Cdk2, (cdk2)*	Cdk2 loss of function	N
6634	*Cdk2, (cdk2)*	UAS Cdk2	S
6631	*Cdk4*	UAS Cdk4	N
6633	*Cyclin A* (*Cyc A*)	UAS Cyc A	N
27718	*Cyclin D (CycD)*	Expresses dsRNA for RNAi of Cyc D (FBgn0010315) under UAS control	N
13203	*tribbles (trbl)*	trbl loss of function	S
4781	*Cyclin E (Cyc E)*	UAS Cyc E	N
30725	*Cyclin E (Cyc E)*	UAS Cyc E	N
10384	*Cyclin E (Cyc E)*	Cyc E hypomorphic	N
6626	*Cyclin B (Cyc B)*	UAS Cyc B	N
6630	*Cyclin B (Cyc B)*	Cyc B loss of function	N
6628	*Cyclin B3 (Cyc B3)*	UAS Cyc B3	S
6635	*Cyclin B3 (Cyc B3)*	Cyc B3 loss of function	N
4557	*Cdk7*	Cdk7 loss of function	N
6639	*Dacapo (dap)*	dap loss of function	N
11377	*Dacapo (dap)*	dap loss of function	N
3499	*wee*	wee hypomorphic	N
4777	*string (stg)*	UAS stg	N
9166	*roughex (rux)*	UAS rux	N
9165	*roughex (rux)*	rux loss of function	N
2492	*fizzy (fzy)*	fzy loss of function	N
11717	*E2F transcription factor(E2f)*	E2F loss of function	N

5553	*DP transcription factor (Dp)*	Dp hypomorphic allele	N
7435	*Retinoblastoma-family protein (Rbf, Rb)*	Rb loss of function	E
16803	*Retinoblastoma-family protein (Rbf, Rb)*	Rb loss of function	E
8342	*Retinoblastoma-family protein (Rbf, Rb)*	Rb loss of function	N
4774	*E2F, Dp*	UAS E2F N, UAS Dp D	E
31501	*Archipelago (ago)*	Expresses dsRNA for RNAi of ago (FBgn0041171) under UAS control.	N
16989	*Archipelago*	loss of function	N
Play role in eye development			
1486	*decapentaplegic (dpp)*	UAS dpp	E
2062	*decapentaplegic (dpp)*	dpp hypomorphic allele	N
2070	*decapentaplegic (dpp)*	dpp ,hypomorphic allele	N
5364	*Epidermal growth factor receptor (EGFR)*	UAS EGFR DN	E
2079	*Epidermal growth factor receptor (Egfr)*	Egfr hypomorphic allele	E
3378	*armadillo (arm)*	arm loss of function	N
4782	*armadillo (arm)*	UAS arm	E
5612	*Delta (Dl)*	UAS Dl	N
Genes that are components of the contractile ring			
11215	*zipper*	zip loss of function	N
25712	*spaghetti squash (sqh)*	sqh loss of function	N
27616	*diaphanous (dia)*	UAS dia	N
4892	*chickadee (chic)*	chic loss of function	N
9234	*twinstar (tsr)*	UAS tsr N	N
Organize and regulate the contractile ring			
11194	*peanut (pnut)*	pnut loss of function	N
4366	*sticky (Sti)*	Sti hypomorphic	N
6671	*Rho-kinase (rok)*	UAS rok	N
11089	*Stretchin-Mlck (Sttrn-Mlck)*	Sttrn-Mlck loss of function	N

11607	*Myosin binding subunit (Mbc)*	Mbc hypomorphic	N
Genes that are important for completion of mitosis			
16103	*Pimples (pim)*	pim loss of function	N
18610	*separase (Sse)*	Sse loss of function	N
RNAi Stocks			
CG Nr	*Stock*		Result
CG5940	*Cyclin A RNAi*		N
CG6376	*E2f RNAi*		N
CG7413	*Rb RNAi*		E
CG 1395	*string RNAi*		N
CG5462	*string RNAi*		N
CG3510	*Cyclin B RNAi*		N
CG4488	*wee RNAi*		N
CG5408	*trbl RNAi*		N
CG4654	*Dp RNAi*		N

7 Abbreviation

AA	Amino Acid
BICP	5-bromo-4-chloro-3-indoly-phosphat
BrdU	Bromdesoxyuridin
C.elegans	Ceanorhabditis elegans
ChiP	Chromatin Immunoprecipitation
Cdks	Cyclin-dependent kinases
CAKs	Cdk-activating kinase
CKIs	Cdk inhibitor proteins
DEPC	Diethylpyrocarbonat
DNA	Desoxyribonukleinsäure
dNTP	Desoxynukleotidtriphosphat
dsRNA	Double Stranded RNA
E. coli	Escherichia coli
EdU	5-ethynyl-2'-deoxyuridine
EDTA	Ethylendiamintetraacetat
FCS	Fetal Calf Serum
FL	Full Length
GMR	Glass Multimer Reporter
Gal-4	GALactose metabolism-4
GST	Glutathion S-Transferase
kb	Kilobase
kDa	Kilodalton
Lac	Lactose
LB	Luria Bertani
MF	Morphogenetic Furrow
mRNA	Messenger RNA
NBT	Nitrobluetetrazoliumchlorid

NLS	Nuclear Localisation Signal
NPR	Non Proliferating Region
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PAGE	Polyacrylamidgelelectoohorese
pre-mRNAs	precursor mRNAs
q-RT-PCR	Quantitative Reverse Transcription PCR
RNA	Ribonucleinsäure
RNAi	RNA Interferens
Rpm	Rotation per minute
RT	Room Temperature
SA-β-gal	Senescence-Associated β-galactosidase
SAHF	Senescence-Associated Heterochromatic Foci
SDS	Sodium Dodocyl Sulfate
S. cerevisiae	Saccharomyces cerevisiae
SMW	Second Mitotic Wave
UAS	Upstream Activation Sequence
X-gal	5-Brom-4-chlor-3-indolyl-ß-D-galactopyranosid

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Statement

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig und ohne Verwendung anderer Hilfsmittel und Hilfern als der in der Arbeit angegebenen verfaßt habe.

Shaza Dehne

Berlin, den 30/ 06/ 2016 …….

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